

The influence of 3 β HSD on adrenal steroidogenesis and the factors which influence its activity

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DECLARATION

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ABSTRACT

This study describes:

- the characterization and comparison of the enzymatic activity of both Angora and ovine 3 β HSD expressed in non-steroidogenic COS-1 cells. The apparent K_m and V_{max} values for the metabolism of PREG, 17-OHPREG and DHEA were determined;
- the characterization of steroid metabolites produced by COS-1 cells coexpressing either Angora or ovine 3 β HSD together with Angora CYP17, in the presence and absence of overexpressed Cyt-b₅, following the metabolism of PREG and 17-OHPREG. 3 β HSD was identified as an additional factor in causing hypocortisolism in the South African Angora goat;
- the influence of Cyt-b₅ on the enzymatic activity of both Angora and ovine 3 β HSD coexpressed in non-steroidogenic COS-1 cells;
- the influence of purified ovine live Cyt-b₅ and anti-Cyt-b₅ IgG on adrenal microsomal 3 β HSD activity. Cyt-b₅ was shown to specifically augment 3 β HSD activity which represents the first documentation of such augmentation in any species;
- the overexpression and purification of Angora 3 β HSD using a baculovirus expression system coupled with a detergent based enzyme purification method;
- the characterization of both substrate and co-factor kinetics for the individual dehydrogenase and isomerase activities of purified 3 β HSD, in the presence and absence of purified ovine liver Cyt-b₅. Cyt-b₅ was shown to increase the affinity of 3 β HSD towards NAD⁺ during the dehydrogenase reaction whilst having no significant influence on the isomerase reaction. This represents the first documentation of Cyt-b₅ influencing co-factor binding in any member of the hydroxysteroid dehydrogenases;
- the FRET analysis of COS-1 cells coexpressing 3 β HSD-eCFP and Cyt-b₅-eYFP fusion proteins, suggesting an allosteric interaction between 3 β HSD and Cyt-b₅.

OPSOMMING

Hierdie studie beskryf:

- die karakterisering en vergelyking van die ensiematiese aktiwiteit van beide Angora en skaap 3β HSD, wat uitgedruk was in nie-steroïed genererende COS-1 selle. Die K_m en V_{max} waardes tydens die metabolisme van PREG, 17-OHPREG en DHEA was bepaal;
- die karakterisering van steroïed metaboliete gegenereer deur COS-1 selle wat Angora of skaap 3β HSD uitdruk saam met Angora CYP17, in die aanwesigheid of afwesigheid van sitochroom b_5 , na die metaboliseering van PREG en 17-OHPREG. 3β HSD was geïdentifiseer as 'n bydraende faktor in die oorsaak van hipokortisolisme in die Suid-Afrikaanse Angorabok;
- die invloed van sitochroom b_5 op die ensiematiese aktiwiteit van beide Angora en skaap 3β HSD wat saam uitgedruk was in nie-steroïed genererende COS-1 selle;
- die invloed van gesuiwerde skaap lewer sitochroom b_5 en sitochroom b_5 teenstof op mikrosomale 3β HSD aktiwiteit. Dit is getoon dat sitochroom b_5 die aktiwiteit van 3β HSD spesifiek verhoog. Hierdie studie verteenwoordig die eerste dokumentasie van so 'n verhoging in enige spesie;
- die uitdrukking en suiwering van Angora 3β HSD deur middel van 'n bakulo-virus sisteem gekoppel aan 'n detergent gebaseerde ensiem suiwerings metode;
- die karakterisering van beide substraat en ko-faktor kinetika vir die afsonderlike dehidrogenase en isomerase aktiwiteite van gesuiwerde 3β HSD, in die aanwesigheid of afwesigheid van gesuiwerde sitochroom b_5 . Dit is getoon dat sitochroom b_5 die affiniteit van 3β HSD teenoor NAD^+ tydens die dehidrogenase reaksie verhoog sonder om 'n beduidende invloed op die isomerase reaksie te hê. Hierdie studie verteenwoordig die eerste dokumentasie van sitochroom b_5 wat ko-faktor binding beïnvloed in enige lid van die hidroksisteroïed dehidrogenase familie van ensieme;
- die analise van FRET sein in COS-1 selle wat beide 3β HSD-eCFP en Cyt- b_5 -eYFP fusie proteïene uitdruk. Die resultate stel voor dat sitochroom b_5 3β HSD aktiwiteit beïnvloed deur middel van 'n allosteriese meganisme.

DEDICATION

In humble obedience to God, who through His grace, saved me and placed my feet on the solid rock of Jesus Christ.

Deut 31:6

“Be strong and of good courage, do not fear nor be afraid... for the Lord your God, He is the One who goes with you. He will not leave you nor forsake you.”

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LIST OF ABBREVIATIONS

11βHSD2	11 β -hydroxysteroid dehydrogenase type 2
17-OHPREG	17-hydroxypregnenolone
17-OHPROG	17-hydroxyprogesterone
3βHSD	3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase
A4	androstenedione
ACAT	acyl-coenzyme A:cholesterol acyltransferase
ACTH	cyclic adenosine monophosphate
ADR	aldo-keto reductase
AF-1	activation function-1 domain
AF-2	activation function-2 domain
AMP	adenosine monophosphate
AP-1	activator protein-1
apo-b₅	cytochrome-b ₅ void of heme
AR	androgen receptor
CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CORT	corticosterone
CYP11A1	cytochrome P450 side-chain cleavage
CYP11B1	cytochrome P450 11 β -hydroxylase
CYP11B2	aldosterone synthase
CYP17	cytochrome P450 17 α -hydroxylase/17,20 lyase
CYP21	cytochrome P450 21-hydroxylase
CYP2B4	cytochrome P450 2B4
CYP3A4	cytochrome P450 3A4
Cyt-b₅	cytochrome-b ₅
DBD	DNA-binding domain
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate

DHT	dihydrotestosterone
DOC	deoxycorticosterone
eCFP	enhanced cyan fluorescent protein
EGF	epidermal growth factor
ENaC	epithelial Na ⁺ channels
ER	endoplasmic reticulum
eYFP	enhanced yellow fluorescent protein
FAD	flavinadenine dinucleotide
FMN	flavinmononucleotide
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
G-6-Pase	glucose-6-phosphatase
GABA	γ -aminobutyric acid receptor
GH	growth hormone
GR	glucocorticoid receptor
GRE	glucocorticoid-response element
HDL	high density-lipoproteins
HMGCoA	3-hydroxy-3-methylglutaryl co-enzyme A
holo-b₅	wild type cytochrome-b ₅
HPA axis	hypothalamic-pituitary-adrenal axis
HSDs	hydroxysteroid dehydrogenases
HSL	hormone-sensitive lipase
HSPs	heat shock proteins
IMM	inner mitochondrial membrane
LBD	ligand-binding domain
LDL	low-density lipoproteins
LH	luteinizing hormone
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor- κ B
NHR	nuclear hormone receptor

NMDA	N-methyl-D-aspartic acid receptor
OMM	outer mitochondrial membrane
P450s	cytochrome P450s
PEPCK	phosphoenolpyruvate carboxykinase
PKA	protein kinase A
POR	cytochrome P450 oxidoreductase
PREG	pregnenolone
PRL	prolactin
PROG	progesterone
ROMK	inwardly rectifying potassium channels
RT-PCR	reverse transcription polymerase chain reaction
SDR	short-chain dehydrogenase/reductase
SF-1	steroidogenic factor-1
SGK-1	serum glucocorticoid-regulated kinase-1
SRBI receptor	scavenger receptor class B, type-I receptor
StAR	steroidogenic acute regulatory protein
START domain	StAR-related lipid transfer domain
Stat	signal transducers and activators of transcription
STATs	signal transducers and activators of transcription
trunc-b₅	truncated cytochrome b ₅
TSH	thyroid-stimulating hormone
TSPO	translocator protein or peripheral benzodiazepine receptor

CHAPTER 1

INTRODUCTION

Steroid hormones play a vital role in mediating a variety of essential physiological processes. In mammals, the adrenal cortex secretes three classes of steroid hormones, namely the glucocorticoids, mineralocorticoids and adrenal androgens. The glucocorticoids are primarily responsible for the regulation of carbohydrate, protein and lipid metabolism whilst also extensively influencing the immune system and inflammatory processes. The mineralocorticoids play an important role in the regulation of sodium reabsorption and water balance whereas the adrenal androgens are primarily involved in regulating sexual development and growth. These hormones are all derived from cholesterol through a network of enzyme catalyzed reactions, collectively known as the steroid hormone biosynthesis pathway. Abnormalities in the catalytic activity of these steroidogenic enzymes which makeup the steroid hormone biosynthesis pathway can result in a number of clinical disorders, including congenital adrenal hyperplasia (CAH) and Cushing's syndrome (Stewart, 2003; Payne and Hales, 2004; Miller and Auchus, 2011 and references therein). Chapter 2 provides an overview of adrenal steroidogenesis to illustrate the vital role steroid hormones play in mediating important physiological processes and how steroidogenic enzymes function to synthesize these hormones.

In the context of adrenal steroidogenesis, one of the most important steroidogenic enzymes is 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β HSD). A member of the hydroxysteroid dehydrogenases, 3 β HSD is a bi-functional membrane-bound enzyme converting Δ^5 steroid intermediates to their corresponding Δ^4 products via sequential dehydrogenase and isomerase reactions. In the first dehydrogenase reaction, NAD⁺ is reduced to NADH with the formation of a Δ^5 3-keto steroid intermediate which, together with NADH, remains bound to the enzyme. NADH subsequently induces conformational changes in the enzyme resulting in the isomerization of the intermediate to form the corresponding Δ^4 steroid product. During adrenal steroidogenesis 3 β HSD catalyzes the conversion of pregnenolone (PREG), 17-hydroxypregnenolone (17-OHPREG) and dehydroepiandrosterone (DHEA) to progesterone (PROG), 17-hydroxyprogesterone (17-OHPROG) and androstenedione (A4), respectively. Consequently, 3 β HSD activity is required for the synthesis of all classes of steroid hormones as PROG, 17-OHPROG and A4 serve as precursors for the synthesis of mineralocorticoids, glucocorticoids, androgens and estrogens (Penning, 1997; Payne and Hales, 2004,

Miller and Auchus, 2011). In this thesis chapter 3 specifically focuses on the physiological importance of 3 β HSD as well as its structure, catalytic mechanism and tissue specific expression.

In addition to playing an essential role in the synthesis of all classes of adrenal steroids, 3 β HSD also competes with cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17) for the steroid intermediates PREG and 17-OHPREG at key branch points in the steroid hormone biosynthetic pathway. As a result the steroidogenic output of the adrenal may be greatly influenced by the relative activity and substrate specificity of this key steroidogenic enzyme (Conley and Bird, 1997; Rainey and Nakamura, 2008; Conley et al., 2011). Indeed, alterations in 3 β HSD activity have been shown to impact various physiological and developmental processes by altering the synthesis of the steroid hormones which mediate these processes. Studies investigating classical 3 β HSD deficiency in humans have shown that a loss in 3 β HSD activity alters the synthesis of cortisol, aldosterone and testosterone (Simard et al., 1995; Morel et al., 1997; Penning, 1997; Pang, 1998; Pang, 2001; Simard et al., 2005 and reference therein). Nevertheless, the manner in which variations in 3 β HSD activity alters the biosynthesis of these hormones has not been fully characterized. At present, limited experimental data are available describing the influence that varying levels of 3 β HSD activity have on the competition between 3 β HSD and CYP17 and how fluctuations in the outcome of this competition ultimately impacts the biosynthesis of active steroid hormones.

The aim of this thesis was therefore firstly, to investigate how variations in 3 β HSD activity influence the competitive role of 3 β HSD in the metabolism of PREG and 17-OHPREG and secondly, to investigate and characterize the factors which influence this competition. However, to effectively investigate the competitive role of 3 β HSD during adrenal steroidogenesis requires the use of a relevant experimental model, one that exhibits a known steroidogenic deficiency that may result from altered 3 β HSD activity levels. To this end the South African Angora goat was chosen as an experimental model.

The South Africa Angora goat is extremely susceptible to cold stress due to a diminished capacity to produce cortisol. Investigations into this susceptibility revealed the relative inability of these animals to regulate their blood glucose levels in response to cold stress, reducing their capacity to produce sufficient metabolic heat, leading to hypoglycaemia (Fourie, 1984; Cronje, 1995; Wentzel et al., 1979). Studies investigating this phenomenon showed that selective breeding for high quality fleece resulted in a reduced adrenal function that is characterized by a decrease in cortisol production (Van Rensburg, 1971; Wentzel et al 1979; Herselman and Loggerenberg, 1995). This was confirmed by Engelbrecht et al (2000) who showed that Angora adrenal steroidogenesis differs significantly from that of the Boer goat and Merino sheep, which are considered more hardy against cold stress. Angora adrenal

microsomal preparations were shown to produce only 35% glucocorticoid precursors compared to 78% and 82% produced by the Boer goat and Merino sheep, respectively. Engelbrecht and Swart (2000) hypothesized that this difference in adrenal steroidogenesis results from differences in the catalytic activity of key steroidogenic enzymes leading to a higher flux of steroid intermediates through the Δ^5 pathway, thereby causing a decrease in cortisol production in the Angora. Subsequent investigation of Angora goat steroidogenic enzyme activity identified 3β HSD and CYP17 as having a potential role in hypocortisolism. A comparative study between the Angora goat, the Boer goat and Merino sheep was conducted which showed Angora CYP17 to have a higher activity towards PREG whilst 3β HSD activity towards PREG was shown to be similar between all three species. It was concluded that an increase in CYP17 activity contributed to hypocortisolism by increasing the flux of steroid intermediates through the Δ^5 pathway, away from cortisol production towards androgen production. Storbeck et al. (2007) later attributed the increase in CYP17 activity to an increase in 17,20 lyase activity towards 17-OHPREG. However, the study conducted by Engelbrecht and Swart (2000) was conducted using adrenal microsomal preparations which not only contained 3β HSD but also various other steroidogenic enzymes and co-factors, complicating the interpretation of data. The contribution of 3β HSD in causing hypocortisolism in the South African Angora goat therefore remained uncertain. Hence, the South African Angora goat provided an excellent model in which to study the influence of 3β HSD activity on adrenal steroidogenesis.

Chapter 4 presents a comparative study in which the cDNA sequence and kinetic parameters of Angora 3β HSD were characterized and compared to ovine 3β HSD as the merino sheep is generally considered more hardy against cold stress. Following characterization, both Angora and ovine 3β HSD were individually coexpressed with Angora CYP17 in COS-1 cells and assayed with PREG and 17-OHPREG as substrate. The resulting steroid metabolites were characterized and compared to ascertain how differences in 3β HSD activity between these species influence the flux of steroid intermediates through the adrenal steroidogenic pathway and ultimately cortisol production. The cDNA sequences were found to differ by five amino acid residues resulting in significant differences between the initial reaction rates of these enzymes towards PREG, 17-OHPREG and DHEA. The initial reaction rate of ovine 3β HSD during the conversion of 17-OHPREG was significantly greater than that of the Angora whilst that of Angora 3β HSD during the conversion of DHEA was significantly greater than that of the ovine enzyme. Coexpression of Angora and ovine 3β HSD with Angora CYP17 revealed that cells expressing Angora 3β HSD produced significantly less 17-OHPROG than cells expressing ovine 3β HSD following the metabolism of PREG and 17-OHPREG. These results, implicating 3β HSD as an additional factor in causing hypocortisolism in the South African Angora goat, were published in

Molecular and Cellular Endocrinology and are presented in the article included in chapter 4 (Goosen et al., 2010).

Furthermore, it is known that the competition between 3β HSD and CYP17 is influenced by cytochrome-b₅ (Cyt-b₅). Cyt-b₅ is a small ubiquitous electron transfer hemoprotein well documented to selectively enhance the lyase activity of CYP17 during the metabolism of 17-OHPREG, resulting in a substantial increase in C19 steroid production (Katagiri et al., 1982; Katagiri et al., 1995; Auchus et al., 1998). Therefore, additional studies were performed to investigate the competitive role of 3β HSD during the metabolism of 17-OHPREG in the presence and absence of overexpressed Cyt-b₅. Results showed that in the absence of overexpressed Cyt-b₅ cells expressing Angora 3β HSD produced significantly less 17-OHPROG compared to cells expressing ovine 3β HSD. However, the presence of overexpressed Cyt-b₅ caused cells expressing Angora 3β HSD to produced significantly higher levels of 17-OHPROG compared to cells expressing ovine 3β HSD. These results, which are presented in chapter 4, could not be accounted for within the bounds of our current understanding of how Cyt-b₅ influences adrenal steroidogenesis and alluded to the possibility that Cyt-b₅ may specifically augment 3β HSD activity.

As no data are currently available describing the influence of Cyt-b₅ on 3β HSD activity, chapter 5 provides a brief background on Cyt-b₅ in the context of its known involvement in various cytochrome P450 catalyzed reactions. This is followed by a study which aimed to determine whether augmentation of 3β HSD activity by Cyt-b₅ exists, and if so, whether such augmentation is specific to 3β HSD. Cyt-b₅ was coexpressed with both caprine and ovine 3β HSD in COS-1 cells and the catalytic activity assayed towards PREG, 17-OHPREG and DHEA. In addition, ovine adrenal microsomes were prepared and 3β HSD activity assayed in the presence and absence of purified Cyt-b₅ and anti-Cyt-b₅ IgG. In COS-1 cells, Cyt-b₅ was shown to augment 3β HSD activity towards all steroid substrates evaluated by between 15 and 25% in a species and substrate specific manner. Furthermore, kinetic studies revealed Cyt-b₅ to have no influence on the apparent K_m values while significantly increasing the apparent V_{max} values towards all three respective steroid substrates. Using a simplified irreversible bi-substrate rate equation, it was shown that by decreasing the K_{NAD}^+ value, the the apparent V_{max} value could be increased without altering the K_m value. In addition, *apo*-b₅ (Cyt-b₅ void of heme) was shown to stimulate 3β HSD activity in COS-1 cells whilst 3β HSD activity in ovine adrenal microsomes was shown to be significantly influenced by either purified Cyt-b₅ or anti-Cyt-b₅ IgG. These data showed Cyt-b₅ to specifically augment 3β HSD activity and led to the hypothesis that the augmentation of 3β HSD activity by Cyt-b₅ is most likely allosteric in nature, resulting in a decreased K_{NAD}^+ value. The results were published in the Journal of Steroid Biochemistry and Molecular Biology and are included

in the article presented in chapter 5 (Goosen et al., 2011) and represents the first documentation of Cyt-b₅ augmenting 3 β HSD activity in any species.

Cyt-b₅ affects a variety of biochemical reactions, including the substrate metabolism of various P450 enzymes involved in xenobiotic metabolism and steroidogenesis. Traditionally, Cyt-b₅ is believed to augment the activity of specific P450s via direct electron transfer. However, a number of studies have implicated Cyt-b₅ in playing an allosteric role in the catalysis of P450 enzymes (Vergères and Waskell, 1995; Porter, 2002; Yamazaki et al., 2002; Schenkman and Jansson, 2003). However, unlike the P450s, 3 β HSD does not require the input of electrons from an external electron donor to perform its catalytic function. In addition, homology modeling of human type I 3 β HSD have previously shown the dehydrogenase and isomerase domains to be linked by a separate shared co-enzyme domain (Thomas et al., 2003). It therefore is plausible that an allosteric interaction could selectively influence NAD⁺ binding without affecting substrate binding.

Chapter 6 presents a study in which we evaluated our previous hypothesis by determining the influence of Cyt-b₅ on the individual dehydrogenase and isomerase activities of 3 β HSD as well as co-factor binding. 3 β HSD was overexpressed in SF-9 cells and purified. Both substrate and co-factor kinetics were independently determined for both the dehydrogenase and isomerase activities using the purified enzyme, in the presence and absence of purified Cyt-b₅. In addition, 3 β HSD-eCFP and Cyt-b₅-eYFP fusion proteins were expressed in COS-1 cells and analyzed for FRET. The data showed the presence of Cyt-b₅ to significantly increase the apparent V_{max} of the first dehydrogenase reaction whilst resulting in a ≈ 3.5 -fold decrease in the K_m value of 3 β HSD towards NAD⁺. In contrast, the presence of Cyt-b₅ had no significant influence on the kinetic parameters of the isomerase reaction. Furthermore, FRET analysis of COS-1 cells coexpressing 3 β HSD-eCFP and Cyt-b₅-eYFP fusion proteins strongly suggested interaction between these fusion proteins in live cells. These data validated our hypothesis and represented the first documentation of Cyt-b₅ influencing co-factor binding in any member of the hydroxysteroid dehydrogenases. These novel findings have been published in the FASEB journal and are included in the article presented in chapter 6 (Goosen et al., 2012).

In conclusion, chapter 7 provides a general discussion of the results obtained in this study with specific focus on the physiological relevance of the major findings.

CHAPTER 2

ADRENAL STEROIDOGENESIS

2.1 Adrenal gland

2.1.1 *Anatomy and morphology*

The adrenals are small endocrine glands, triangular in shape, located on the superior poles of the kidneys. In mammals, including sheep and Angora goats, the adrenal gland can be divided into two anatomically and functionally different endocrine tissues, namely the steroid producing cortical cells and the catecholamine producing chromaffin cells. Conventionally, these endocrine cells are separated into an outer cortex and an inner medulla (Fig 2.1 A) (Ehrhart-Bornstein et al., 1998; Brook and Marshall, 2001; Stewart, 2003). However, chromaffin cells of the medulla have been found in all zones of the adrenal cortex, either radiating through the cortex from the medulla or forming islets in the cortex (Palacios and Lafraga, 1975; Gallo-Payet et al., 1987; Bornstein et al., 1991). Conversely, cortical cells have also been found in the medulla where they form islets that are completely surrounded by cortical tissue or retain some contact with the rest of the cortex (Bornstein et al., 1991, 1994).

The adrenal medulla, which constitutes approximately 10-20% of the adrenal volume, is made up of chromaffin cells that originate from neural crest precursor cells. These cells migrate into the adrenal and under the influence of adrenocortical steroids subsequently differentiate into chromaffin cells (Ehrhart-Bornstein et al., 1998; Stewart, 2003). Chromaffin cells are packed closely into clusters supported by a collagenous network and are characterized by large nuclei and an extensive, granular cytoplasm containing no stored lipids. The main secretory products of these cells are the catecholamines, epinephrine and norepinephrine, as well as numerous transmitters and neuropeptides (Young and Heath, 2002).

The outer cortex serves as the primary steroid producing tissue and constitutes approximately 80-90% of the adrenal volume. The cortex is formed from mesenchymal cells attached to the coelomic cavity lining adjacent to the urogenital ridge. In the human fetus, two distinct adrenal zones can be identified, the inner fetal zone and an outer definitive zone, with the latter differentiating into the adult adrenal. The adrenal cortex secretes three classes of steroid hormones, namely the mineralocorticoids, glucocorticoids and adrenal androgens (Mesiano and Jaffe, 1997; Jaffe et al., 1998; Stewart, 2003).

The steroid secreting cells of the cortex are characterized by an abundant cytoplasm containing large lipid droplets, variably shaped mitochondria and an extensive system of smooth endoplasmic reticulum (Fig 2.2). In most mammals, including sheep and Angora goats, these cells form three distinct zones which vary in morphological features and steroid hormone production. These zones, arranged in concentric layers around the inner medulla are, from the outside, the zona glomerulosa, zona fasciculata and the zona reticularis (Fig 2.1 B) (Young and Heath, 2002).

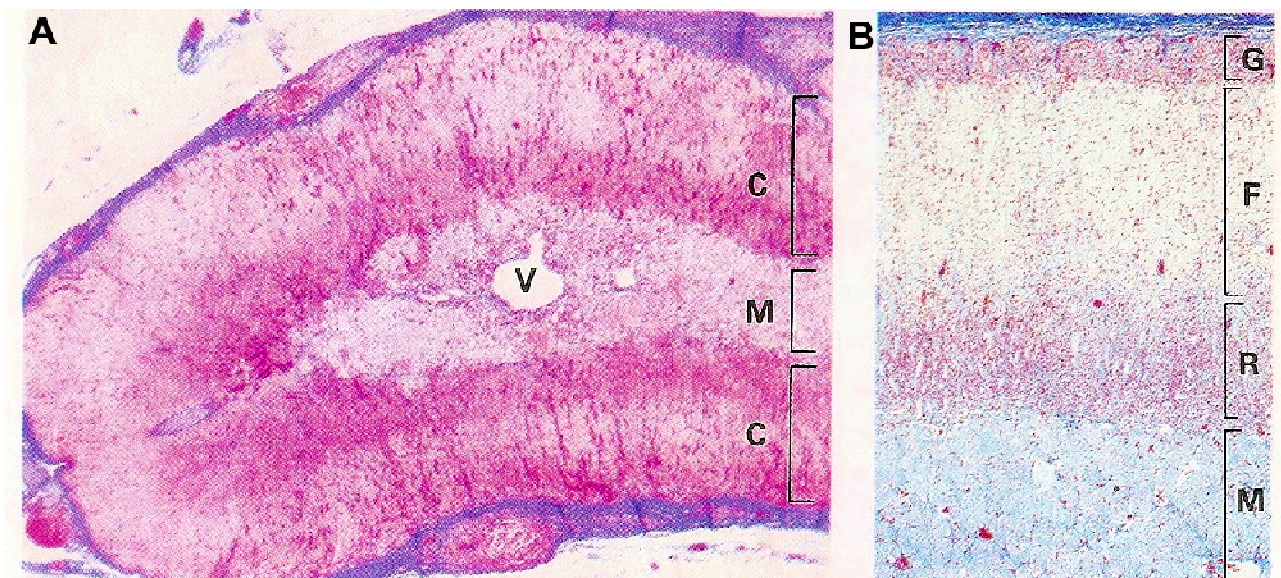


Figure 2.1 **A:** Cross-section of the adrenal gland showing the inner medulla (**M**) surrounded by the outer cortex (**C**) and a prominent vein (**V**) in the centre of the medulla. **B:** Cross-section of the adrenal cortex and medulla (**M**) showing the three histological zones of the adrenal cortex: the zona glomerulosa (**G**), zona fasciculata (**F**) and zona reticularis (**R**). Reproduced from Young and Heath (2002).

The outer most zona glomerulosa lies directly beneath the fibrous capsule that surrounds the adrenal and makes up approximately 5-10% of the cortical volume (Brook and Marshall, 2001). The cells in this layer are arranged in irregular ovoid clusters and secrete the mineralocorticoid, aldosterone (Young and Heath, 2002). Furthermore, this zone also appears to generate new fasciculata and reticularis cells through a progenitor cell population lying between the zona glomerulosa and zona fasciculata (Neville and O'Hare, 1985; Steward, 2003). The zona fasciculata, which forms the middle cortical zone, constitutes approximately 75% of the volume of the cortex. Cells in this layer secrete the glucocorticoids, cortisol and corticosterone as well as trace amounts of the adrenal androgen dehydroepiandrosterone (DHEA) (Brook and Marshall, 2001). Cells form narrow, radially arranged

cords separated by fine strands of supporting tissue containing wide capillaries. The thin innermost zone of the cortex is the zona reticularis which consists of an irregular network of branching cords and clusters of glandular cells separated by numerous capillaries. These cells, which are significantly smaller than the cells of the zona fasciculata, contain fewer lipid droplets and primarily produce the adrenal androgens DHEA, DHEA sulphate (DHEAS) and androstenedione (A4) as well as trace amounts of glucocorticoids (Brook and Marshall, 2001; Young and Heath, 2002).

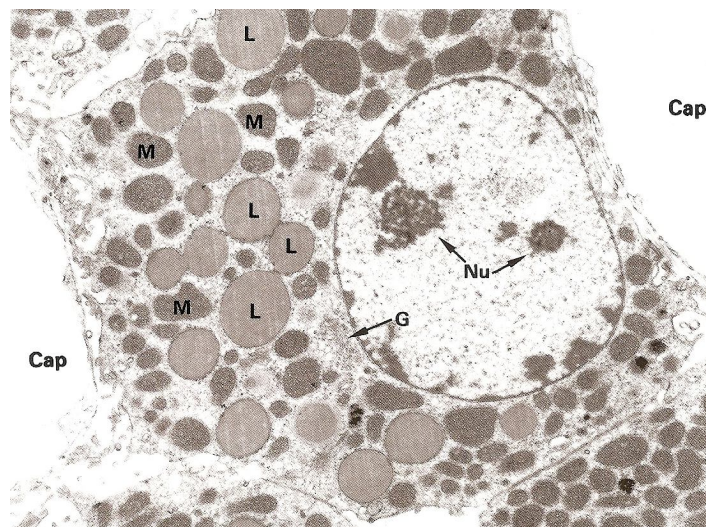


Figure 2.2 Micrograph (EM $\times 8500$) illustrating the typical ultrastructure of a steroid-secreting cell in the adrenal cortex. The round nucleus contains a number of prominent nucleoli (Nu). The cell is characterized by the cytoplasm containing a number of lipid droplets (L) and numerous variably shaped mitochondria (M). A small Golgi apparatus (G) can also be seen near the nucleus. Reproduced from Young and Heath (2002).

2.2 Hormones of the adrenal cortex

The mineralocorticoids, glucocorticoids and adrenal androgens, produced by the adrenal, play an important role in mediating a variety of physiological processes. The mineralocorticoid aldosterone plays an important role in the regulation of sodium reabsorption in the kidney and from saliva and sweat whilst also being involved in the regulation of electrolyte concentrations in the extracellular fluid. The glucocorticoids mediate lipid, protein and carbohydrate metabolism whereas the adrenal androgens are primarily involved in regulating sexual development and growth (Farman and Rafestin-Oblin, 2001; Steward, 2003; Meneton et al., 2004; Widmaier, Raff and Strang, 2004). The general mechanism of action as well as the various physiological effects of the adrenocortical steroids will be

discussed in the following section, highlighting the vital role these hormones play in maintaining normal physiological processes.

2.2.1 Mechanism of action

Steroid hormones are lipophilic and it is assumed that these hormones enter target cells by simple diffusion (Falkenstein et al., 2000). Inside the target cell, steroid hormones bind to intracellular hormone receptors, leading to altered expression of specific hormone-responsive target genes. This mechanism is termed “genomic” as it mediates changes in gene expression (Beato et al., 1996; Nicolaides et al., 2010). In addition to this classic genomic action of steroid hormones, non-genomic actions have also been documented which are considered to have no direct effects on gene expression. (Reviewed in Falkenstein et al., 2000; Funder, 2005; Good, 2007). These non-genomic effects are, however, beyond the scope of this discussion and it is on the classic genomic actions of steroid hormones alone that this thesis will focus.

In the cytoplasm of the target cell, steroid hormones bind to intracellular proteins known as nuclear hormone receptors (NHR). These proteins belong to the steroid/thyroid/retinoic acid nuclear receptor superfamily of transcription factors which mediate the expression of hormone-responsive genes, positively or negatively (Gustafsson et al., 1987; Steward, 2003; Nicolaides et al., 2010). The NHRs for each of the major classes of adrenocortical steroids have been identified and cloned. These are the mineralocorticoid receptor (MR) (Arriza et al., 1987), the glucocorticoid receptor (GR) (Hollenberg et al., 1985) and the androgen receptor (AR) (Chang et al., 1988; Lubahn et al., 1988). As the mechanisms of action of these receptors are similar and since the GR has been characterized most extensively, this receptor will be discussed further.

Two isoforms of the human GR have been identified and are formed by alternative splicing of the gene encoding the GR in exon 9 (Encio and Detera-Wadleigh, 1991; Lu and Cidlowski, 2005). These are termed GR α and GR β . GR α represents the classic glucocorticoid receptor that functions as a ligand-dependant transcription factor. In contrast, GR β does not bind glucocorticoid agonists and has an intrinsic dominant negative effect upon the transcriptional activity of GR α (Oakley et al., 1999; Zhou and Cidlowski, 2005; Duma et al., 2006). Only the ligand-dependant genomic effects of GR α will be discussed further.

The GR α is structurally organized into different domains. These include the A/B regions which comprise the N-terminal domain, the C, D and E regions corresponding to the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LDB), respectively (Falkenstein et al., 2000;

Nicolaides et al., 2010). A major transactivation domain, known as activation function-1 (AF-1), is located in the N-terminal domain between amino acid residues 77 and 262. This domain plays an important role in the interaction of the GR with other coactivators, chromatin modulators and basal transcription factors necessary for the initiation of transcription. The most conserved domain throughout the NHR family is the DBD. The DBD of the GR α is located between amino acid residues 420 and 480 and contains two zinc finger motifs. The GR α uses these motifs to bind to specific DNA sequences, known as glucocorticoid-response elements (GREs), located in the promoter region of hormone-responsive genes. The variable region between the DBD and the LBD is known as the hinge region. This region is involved in the dimerization of the DBD and also confers structural flexibility to the receptor dimers, allowing a single receptor dimer to interact with multiple GREs. The LBD is located between amino acid residues 481 and 777 and results in ligand-induced activation of the GR α upon binding of glucocorticoid hormone. This domain also contains a second functional domain, termed activation function-2 (AF-2), involved in nuclear translocation, binding of heat shock proteins (HSPs) and receptor dimerization (Chrousos, 2004; Zhou and Cidlowski, 2005; Duma et al., 2006; Nicolaides et al., 2010 and references therein).

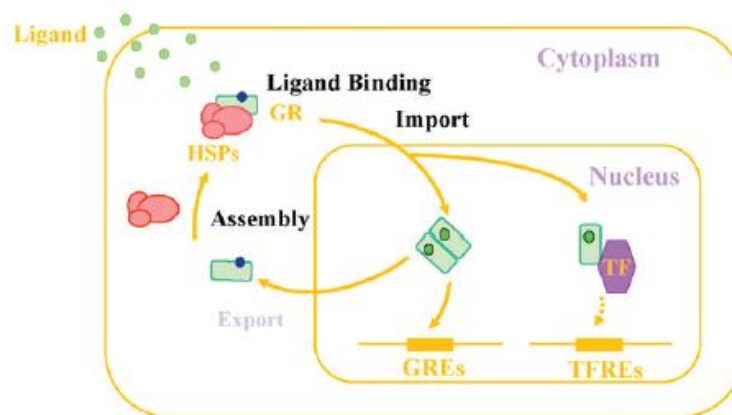


Figure 2.3 Diagrammatic representation of gene activation via the GR. Upon ligand binding the GR dissociates from HSPs and translocates into the nucleus. In the nucleus the GR-ligand complex binds either to GREs or associate with other transcription factors (TF). Reproduced from Nicolaides et al. (2010).

The GR α resides in the cytoplasm of target cells primarily as part of a hetero-oligomeric complex in the absence of ligand (Fig 2.3). This complex is made up of chaperone HSPs 90, 70, 50, immunophilins as well as other proteins. HSPs regulate ligand binding and cytoplasmic retention of the

receptor whilst immunophilins are thought to be involved in the regulation of GR signalling (Pratt, 1993; Steward, 2003; Nicolaides et al., 2010). Upon ligand binding, the receptor undergoes a conformational change resulting in its dissociation from the multi-protein complex and subsequent translocation into the nucleus (Pratt, 1993; Terry et al., 2007). In the nucleus, the receptor-ligand complex binds as homodimers to GREs located in the promoter region of hormone-responsive target genes. Binding of the GREs results in either positive or negative regulation of the target gene, depending on the GRE and the promoter context (Fig 2.3) (Bamberger et al., 1996; Schaaf and Cidlowski, 2002). Alternatively, the ligand-activated GR α can modulate gene expression without directly binding to GREs. This is thought to be accomplished by the monomeric GR α -ligand complex through interactions with transcription factors, such as activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B) and other signal transducers and activators of transcription (STATs) (Jonat et al., 1990; Scheinman et al., 1995; Kino and Chrousos, 2002). Following transcriptional modulation of hormone-responsive genes the GR α dissociates from the ligand and the GRE. The unliganded GR α is then exported to the cytoplasm where it may be recycled or degraded in the proteasome (Liu and DeFranco, 2000; Nicolaides et al., 2010).

2.2.2 Glucocorticoids

Glucocorticoids are primarily responsible for the regulation of carbohydrate, protein and lipid metabolism. The adrenal cortex produces two steroid hormones that exhibit glucocorticoid activity, namely cortisol and corticosterone. One of these normally functions as the primary glucocorticoid within a species and is secreted at much higher levels compared to the other. In humans and goats, cortisol is the primary glucocorticoid accounting for approximately 95% of glucocorticoid activity exhibited by adrenocortical hormones (Guyton and Hall, 2000; Steward, 2003; Widmaier et al., 2004).

The regulatory actions of glucocorticoids on metabolism may have permissive and stress-associated components that synergize with, or suppress the effects of other hormones such as glucagon, catecholamines, growth hormone (GH) and insulin (Sapolsky et al., 2000). To illustrate the regulatory actions of glucocorticoids the effect of cortisol on blood glucose levels in response to physiological stress will be discussed.

In mammals, physiological stress (such as cold stress) results in a drop in blood glucose levels as glucose is the primary energy source for thermogenesis via shivering. In response to changes in core body temperature as well as low blood glucose levels the adrenal cortex is stimulated to secrete cortisol through the hypothalamic-pituitary-adrenal (HPA) axis (Munch, 1971; Guyton and Hall, 2000;

Sapolsky et al., 2000; Pacak and Polkovits, 2001). The subsequent rise in plasma cortisol concentration results in an increase in blood glucose levels through cortisol action on glycogen as well as protein and lipid metabolism (Guyton and Hall, 2000; Widmaier et al., 2004). In the liver, cortisol induces an increase in glucose output through the activation of several key enzymes involved in gluconeogenesis via transcriptional activation. Principal among these are the two so-called “rate-limiting” enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) (Exton, 1987; Pilkis and Granner, 1992). Glycogen synthesis in the liver is also stimulated by an increase in glycogen synthase activity and inhibition of glycogen phosphorylase activity (von Holt and Fister, 1964; Stewart, 2003). Cortisol further regulates blood glucose levels by decreasing glucose uptake and utilization in several peripheral tissues whilst also activating protein catabolism and lipolysis (Olefsky, 1975; Carter-Su and Okamoto, 1987; Sapolsky et al., 2000). The effects of cortisol therefore synergize with those of glucagon whilst suppressing those of insulin (Stewart, 2003; Widmaier et al., 2004)

Abnormal glucocorticoid synthesis results in a number of physiological effects. In humans, patients who suffer from cortisol deficiency may present with vague features of chronic adrenal insufficiency such as weakness, weight loss, tiredness and general malaise. In addition, cortisol deficiency may also substantially impair the body’s ability to regulate blood glucose levels in response to physiological stress (Stewart, 2003). Nowhere is this more apparent than in the South African Angora goat.

The South Africa Angora goat is extremely susceptible to cold stress resulting in severe stock losses during cold spells (Terblanche, 1987). Investigations into this susceptibility revealed the relative inability of these animals to regulate their blood glucose levels in response to cold stress. This inability reduces the capacity of these animals to produce sufficient metabolic heat, ultimately leading to hypothermia and death (Fourie, 1984; Cronje, 1995; Wentzel et al., 1979). Studies investigating this phenomenon showed that selective breeding for high quality fleece resulted in a reduced adrenal function that is characterized by a decrease in cortisol production (Van Rensburg, 1971; Wentzel et al 1979; Herselman and Loggerenberg, 1995). This was later confirmed by Engelbrecht et al (2000) who showed that *in vivo* stimulation of the Angora HPA axis with insulin and ACTH resulted in lower cortisol production by Angora goats, relative to other more hardy ruminant species. Therefore, the inadequate production of cortisol by the Angora’s adrenal glands in response to physiological stress levels severely impairs its ability to cope with cold conditions.

In addition to their actions on metabolism, glucocorticoids also extensively influence the immune system and inflammatory processes. Elevated glucocorticoid levels, as seen during prolonged periods of physiological stress, suppress immunologic responses. In blood, lymphocyte counts are reduced by

the redistribution of lymphocytes from the intravascular compartment to the spleen, lymph nodes and bone marrow (Yu et al., 1974; Stewart, 2003). T and B lymphocytes are also directly influenced through inhibition of immunoglobulin biosynthesis and stimulation of lymphocyte apoptosis (Cidlowski et al., 1996; Stewart, 2003). The production and activity of cytokines, such as IL-1, IL-2, IL-3 and IL-8, are also inhibited through the repression of cytokine gene transcription via the inhibition of NF- κ B activity. Glucocorticoids inhibit the function of NF- κ B by either binding directly to it or by inducing NF- κ B inhibitor (Munck and Náray-Fejes-Tóth, 1994; Barnes, 1998; Sapolsky et al., 2000; Stewart, 2003). Additional anti-inflammatory effects of glucocorticoids include inhibition of monocyte differentiation into macrophages and the inhibition of histamine and plasminogen activators (Stewart, 2003).

Furthermore, prolonged exposure to elevated glucocorticoid levels results in a number of symptoms and signs collectively termed Cushing's syndrome. Symptoms and clinical features of Cushing's syndrome include weight gain and obesity (Wajchenberg et al., 1995), psychiatric dysfunction (Jeffcoate et al., 1979), muscle weakness (Pleasure and Engel, 1970), skin thinning (Ferguson et al., 1983), hypertension, osteoporosis and diabetes (Ross and Linch, 1982; Stewart, 2003). Gonadal dysfunction associated with menstrual irregularity in females and loss of libido in both sexes is common. Hirsutism and acne is also frequently observed in female patients (Chrousos et al., 1998; Stewart, 2003). Patients who suffer from Cushing's syndrome are also more susceptible to infections due to the suppression of normal immune and inflammatory responses, as described above. Furthermore, prolonged exposure to elevated cortisol levels also cause significant catabolic changes in peripheral tissues. In the skin cortisol inhibits epidermal cell division and collagen synthesis whilst causing atrophy and reduced protein synthesis in muscle tissue (Stewart, 2003). Cortisol also influences bone and calcium metabolism by inhibiting osteoblast function and inhibiting intestinal calcium absorption and stimulating renal calcium excretion (Canalis, 1996; Manolagas, 2000). The physiological consequences of prolonged elevated glucocorticoid levels on various parts of the body are summarized in Fig 2.4.

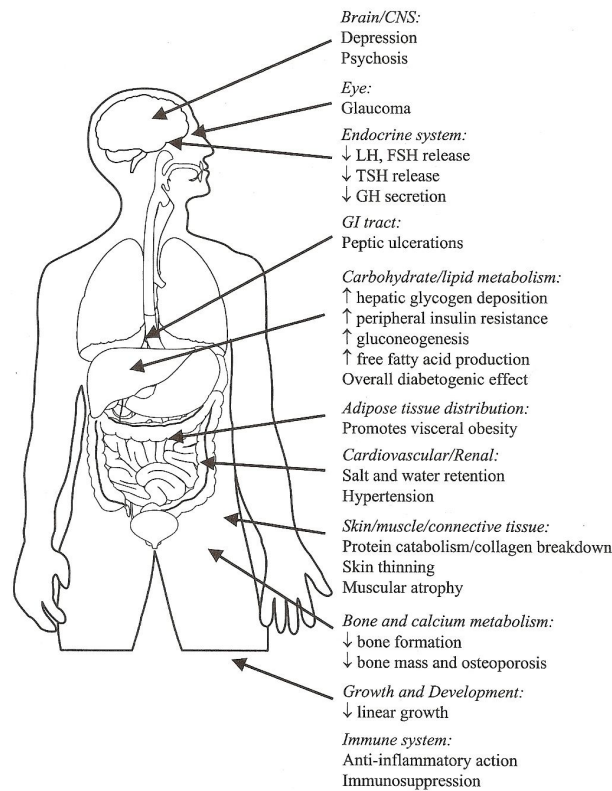


Figure 2.4 Summary of the main consequences of glucocorticoid excess in the human body. CNS, central nervous system; GI, gastrointestinal; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone. Reproduced from Stewart (2003).

2.2.3 Mineralocorticoids

The mineralocorticoid aldosterone plays a pivotal role in regulating electrolyte and fluid homeostasis. This regulation is achieved through stimulation of Na^+ absorption and K^+ secretion by epithelia in the kidney, colon and salivary glands (Rossier and Palmer, 1992; Booth et al., 2002; Good, 2007 and references therein).

In the kidney such effects occur in the distal tubule, the connecting tubule and collecting duct of the renal nephron where Na^+ is transported through epithelial cells into the interstitial fluid. An electrochemical gradient permits the passage of sodium from the lumen into principal epithelial cells through epithelial Na^+ channels (ENaC) located in the apical membrane. Sodium is then actively pumped out of the cell into the interstitial fluid and the blood by Na^+-K^+ ATPases located in the basolateral membrane, with water following the movement of sodium. The potassium that enters the cell via Na^+-K^+ ATPases is in turn secreted through the inwardly rectifying potassium channels

(ROMK) located in the apical membrane (Rossier and Palmer, 1992; Bonvalet, 1998; Meneton et al., 2004; Good, 2007; Connell et al., 2008).

Aldosterone stimulates sodium absorption by increasing ENaC activity. This is achieved by changes in gene transcription via the mineralocorticoid receptor (MR), resulting in the induction of regulatory proteins leading to an increase in the activity and number of functional ENaC channels in the apical membrane (Rossier and Palmer, 1992; Loffing et al., 2001; Lifton et al., 2001; Good, 2007). In addition to stimulating ENaC activity, aldosterone also stimulates Na⁺-K⁺ ATPase activity through transcriptional regulation. This results in the coordinated regulation of sodium absorption by increasing the transport of sodium across both the apical and basolateral membranes (Loffing et al., 2001; Summa et al., 2001; Good, 2007; Connell et al., 2008). Aldosterone may also stimulate ROMK activity by inducing the expression of serum glucocorticoid-regulated kinase-1 (SGK-1) and protein kinase A (PKA) (Yoo et al., 2003). However, it appears that ROMK activity is primarily controlled by dietary potassium intake (Meneton et al., 2004). Furthermore, aldosterone is also responsible for maintaining acid-base homeostasis. This is achieved in the collecting duct of the renal nephron through proton secretion via vacuolar H⁺-ATPases. Here aldosterone influences net acid secretion through genomic regulation of the apical H⁺-ATPases as part of the final step in urine acidification (Alpern, 2000; Good, 2007).

Abnormal aldosterone synthesis results in a variety of physiological effects through its actions on epithelial cells. Insufficient aldosterone secretion causes salt wasting and water loss leading to extracellular fluid dehydration and low blood volume. Aldosterone deficiency also results in elevated potassium levels in the extracellular fluid, which may lead to cardiac toxicity and the development of cardiac arrhythmia. In contrast, excessive aldosterone secretion results in potassium depletion and elevated sodium and water reabsorption, causing an increase in extracellular fluid volume and hypertension. Excessive aldosterone secretion may also cause a condition known as hypokalemia, which results from a substantial decrease in the plasma potassium concentration causing severe muscle weakness (Guyton and Hall, 2000). In addition, aldosterone excess, particularly in the presence of a high salt intake, may also exert adverse effects on non-epithelial cells in the kidney, blood vessels and heart (Briet and Schiffrin, 2010). These effects include inflammation, fibrosis and podocyte injury in the kidney and cardiovascular remodelling and fibrosis in the cardiovascular system (Greene et al., 1996; Young et al., 1994; Blasi et al., 2003; Briet and Schiffrin, 2010).

2.2.4 Adrenal androgens

The adrenal zona reticularis produces a number of C19 steroids that are commonly referred to as adrenal androgens but do not necessarily exhibit androgenic activity. These androgens serve primarily as precursors for more potent sex steroids such as testosterone and estrogens during sexual development and also promote protein anabolism in target tissues (Haning et al., 1985; Labrie et al., 1998; Burger, 2002). The androgens produced by the adrenal cortex under basal conditions and ACTH stimulation are androstenedione (A4), dehydroepiandrosterone (DHEA) and its sulphated derivative DHEA-sulphate (DHEAS), 11 β -hydroxyandrostenedione and testosterone (Guyton and Hall, 2000; Xing et al., 2011). Here only the effects of DHEA and DHEAS will be discussed as these have the highest serum concentration of the C19 steroids produced by the adrenal (Burger, 2002; Buvat, 2003; Panjari and Davis, 2010).

In the human body DHEAS has the highest serum concentration of all steroid hormones. One of the major characteristics of these hormones is their age-dependant pattern of secretion. During early fetal development both DHEA and DHEAS are produced but drop to very low levels after birth and only increases again from the age of 6-9 years with the highest serum levels occurring between the ages of 15 and 45 years. Thereafter there is a steady decline in production with the residual serum concentration of these hormones in elderly people between the ages of 80-90 years being only 10-20% of that observed in young adults (Orentreich et al., 1992; Buvat, 2003). The increase in DHEA and DHEAS production at around 6-9 years of life is known as adrenarche and represents the best known correlation between the synthesis of these hormones and physiological effects. Adrenarche is defined as the developmental increase in the adrenal secretion of DHEA and DHEAS several years before gonadal maturation (Weber et al., 1997). Adrenarche is thought to play a role in secondary sexual differentiation such as the development of pubic and axillary hair, functional maturation of apocrine glands and the appearance of acne (Stewart et al., 1992; Ishihara et al., 1993). However, the exact role of DHEA and DHEAS in mediating these and other physiological processes is poorly understood (Weber et al., 1997).

Conventionally, DHEA and DHEAS were considered as pro-hormones with no direct hormonal effects (Burger, 2002; Simpson, 2002; Buvat, 2003). This was partially due to two reasons. Firstly, these hormones exhibit a much lower affinity for the androgen receptor than testosterone and secondly, no DHEA specific hormone receptor has been identified. Therefore, DHEA and DHEAS were considered weak androgens that exert indirect androgenic effects following their conversion to more potent sex steroids in peripheral target tissues (Beck and Handa, 2004).

However, this concept changed with the discovery of a putative specific DHEA receptor on the plasma membrane of bovine aortic endothelial cells (Liu and Dillon, 2002). This suggests the presence of a possible intracellular DHEA receptor, since all major steroid hormones for which membrane receptors have been identified also have well characterized intracellular receptors (Buvat, 2003). This is further supported by data indicating the existence of a putative DHEA specific receptor in human vascular muscle cells (Williams et al., 2002). In addition, other direct effects of DHEA and DHEAS have also been observed such as inhibition of the γ -aminobutyric acid (GABA)_A receptor (Sousa and Ticku, 1997) and modulation of the N-methyl-D-aspartic acid (NMDA) and σ -1 receptors in the rodent brain (Johansson and Le Greves, 2005; Cheng et al., 2008). DHEA has also been shown to have antiapoptotic effects in the human and bovine vascular endothelium (Liu et al., 2007) and neuroprotective effects in human neural stem cells (Suzuki et al., 2004; Bhagra et al., 2008). Together these data suggest that, in addition to serving as precursors for more potent sex steroids, DHEA and DHEAS may also have direct hormonal effects. However, the physiological implication of DHEA and DHEAS exerting direct hormonal effects is still uncertain. A number of studies have suggested beneficial effects for DHEA in the cardiovascular system, in memory function, sexual function and ageing. However, these effects remain to be thoroughly validated (Buvat, 2003; Bhagra et al., 2008; Panjari and Davis, 2010).

2.3 Cholesterol: Precursor for steroid hormone biosynthesis

During adrenal steroidogenesis, cholesterol serves as precursor for the synthesis of all adrenal steroid hormones, hence steroid hormones have closely related structures based on the cyclopentanophenanthrene ring depicted in figure 2.5. The biosynthesis of these steroids is initiated at the inner mitochondrial membrane (IMM), which is relatively poor in cholesterol. This necessitates the transport of cholesterol to the mitochondria in order to sustain adequate levels of steroid hormone production (Rone et al., 2009; Miller and Auchus, 2011 and references therein). Sources of cholesterol and intracellular transport mechanisms prior to its utilization in steroidogenesis will be discussed in the following section.

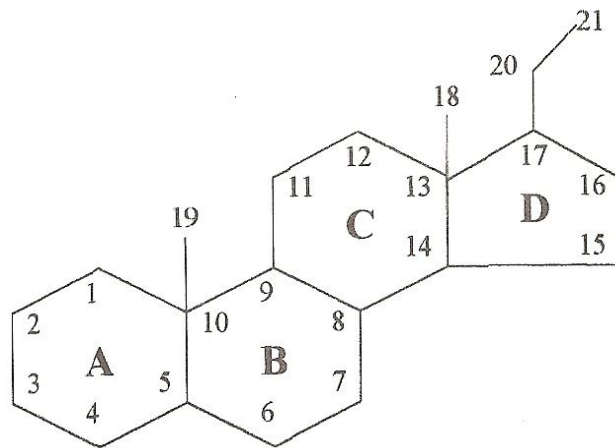


Figure 2.5 Cyclopentanophenanthrene ring structure present in all steroid hormones. Reproduced from Stewart (2003).

2.3.1 Cholesterol uptake and transport to the outer mitochondrial membrane

In the adrenal cortex, most of the cholesterol needed for steroidogenesis is provided from circulation in the form of lipoproteins derived from dietary cholesterol. The remainder is synthesized *de novo* from acetate in the endoplasmic reticulum (ER) or mobilized from other intracellular sources (Gwynne and Strauss, 1982; Mason and Rainey, 1987; Stewart, 2003; Miller and Auchus, 2011).

Adrenal cortical cells obtain cholesterol through the utilization of two forms of lipoproteins present in circulation. These are low-density lipoproteins (LDL) and high density-lipoproteins (HDL), with the uptake of each mediated by a specific cell surface receptor. The uptake of LDL is mediated by the LDL-receptor through receptor-mediated endocytosis (Faust et al., 1977; Stewart, 2003; Rone et al., 2009). The resulting endosome fuses with lysosomes containing acid lipase which subsequently hydrolyzes LDL to produce free cholesterol (Ungewickell and Hinrichsen, 2007). The resulting free cholesterol can be used for steroid hormone biosynthesis or esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT) and stored in cytoplasmic lipid droplets (Chang et al., 1997). Cholesterol esters stored in cytoplasmic lipid droplets can be converted back into free cholesterol by hormone-sensitive lipase (HSL) upon demand (Brown et al., 1979; Rone et al., 2009; Miller and Auchus, 2011). HDL uptake is mediated by the scavenger receptor class B, type-I receptor (SRBI). Unlike the LDL receptor in which the LDL particle is absorbed, the SRBI receptor forms a hydrophobic channel allowing the incorporation of cholesterol esters directly into the plasma membrane (Acton et al., 1996; Connelly and

Williams, 2003). These cholesterol esters are in turn converted to free cholesterol by HSL or stored in lipid droplets (Fig 2.6) (Kraemer and Shen, 2002; Connelly et al., 2003; Rone et al., 2009).

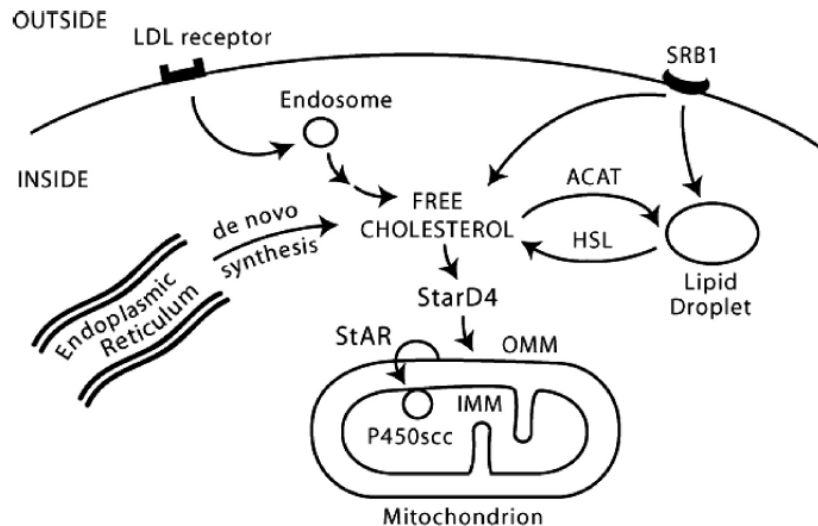


Figure 2.6 Principal pathways of intracellular cholesterol transport. The diagram depicts LDL, HDL and ER as principal sources of free intracellular cholesterol. The transport of free cholesterol to the IMM is facilitated by StarD4 and StAR where it is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450_{scc}). Reproduced from Miller and Auchus (2011).

Another source of intracellular cholesterol is the *de novo* synthesis of free cholesterol from acetate (Mason and Rainey, 1987). This occurs in the ER with the rate-limiting enzyme being 3-hydroxy-3-methylglutaryl co-enzyme A (HMGCoA) reductase (Fig 2.6). The cholesterol generated in the ER may be translocated to the mitochondria via the golgi apparatus or by passive diffusion through contact sites between the ER and the outer mitochondrial membrane (OMM) (Rone et al., 2009 and references therein). The transport of free cholesterol to the OMM is, however, mainly mediated by binding proteins. These proteins form part of a family of proteins that contain StAR-related lipid transfer (START) domains, which include StarD4, -5 and -6. They lack the signalling sequences needed for targeting to specific subcellular organelles and appear to be confined to the cytoplasm. In the cytoplasm these proteins bind to cholesterol and mediate its transport across the aqueous cytosol to the OMM (Soccio and Breslow, 2003; Miller and Auchus, 2011).

The level of intracellular free cholesterol appears to be regulated by LDL concentrations and ACTH. Adequate intracellular LDL concentrations suppress HMGCoA reductase, whilst ACTH

stimulates the activity of HMGCoA reductase, LDL-receptors and LDL uptake. ACTH also stimulates HSL activity and inhibits ACAT activity, thereby increasing the availability of free cholesterol for steroidogenesis (Miller and Auchus, 2011).

2.3.2 Cholesterol transport across the mitochondrial inter-membrane space

Following the transport of cholesterol to the OMM, the cholesterol remains isolated in the OMM prior to being translocated to the IMM where the first enzymatic step of steroidogenesis occurs (Rone et al., 2009; Miller and Auchus, 2011). Since a rapid secretion of steroid hormones in response to stress requires the rapid synthesis of new steroid and the diffusion of cholesterol across this aqueous inter-membrane space occurs very slowly, it was proposed that the translocation of cholesterol across the inter-membrane space is mediated by the steroidogenic acute regulatory protein, StAR (Stocco and Sodeman, 1991; Clark et al., 1994; Stocco and Clark, 1996; Stocco, 2001; Miller and Auchus, 2011).

StAR is synthesized as a 37-kDa protein containing a mitochondrial leader sequence that targets it to the mitochondrion. Upon mitochondrial entry, the leader sequence is cleaved off to yield a 30-kDa intra-mitochondrial protein (Jefcoate, 2002; Miller and Auchus, 2011). StAR's mechanism of action has been studied extensively but is still not fully understood. There has been some uncertainty as to which of the StAR forms represents the biologically active form and whether it acts on the outer or inner mitochondrial membrane (Miller, 2007; Miller and Auchus, 2011). Initially the 30-kDa intra-mitochondrial form was thought to be the biologically active form due to its longer half-life. However, when both the 37-kDa and 30-kDa forms were expressed in the cytoplasm, both were found to be active (Arakane et al., 1996). In addition, the immobilization of StAR on the OMM showed that it was constitutively active but inactive when localized to the inter-membrane space and the IMM (Bose et al., 2002). These data suggest that StAR is only active on the OMM and that it is StAR's cellular localization which determines biological activity and not the cleavage thereof (Miller and Auchus, 2011).

StAR is the first described member of the StAR-related lipid transfer (START) domain family of proteins implicated in lipid and cholesterol transport. The START domain is a 210 amino acid residue motif, suggested to play a role in lipid and cholesterol binding. This domain forms a beta sheet core surrounded by two alpha helices that forms a hydrophobic pocket which is able to accommodate a single cholesterol molecule (Strauss et al., 2003; Alpy and Tomasetto, 2005). StAR's interaction with the OMM has been suggested to involve conformational changes necessary to accept and discharge cholesterol molecules using this domain (Bose et al., 1999; Baker et al., 2005). StAR has been shown

to transfer cholesterol molecules between synthetic membranes *in vitro*. However, the biologically inactive mutant, R182L, is also capable of performing this function, suggesting StAR's ability to promote steroidogenesis is distinct from its cholesterol-transfer activity (Tuckey et al., 2002; Baker et al., 2007).

Furthermore, the action of StAR to promote steroidogenesis has also been shown to require the presence of other proteins such as the translocator protein, TSPO (also known as the peripheral benzodiazepine receptor) in the OMM. It appears that StAR interacts with TSPO, voltage-dependant anion channel-1 and phosphate carrier proteins in the OMM to move a number of cholesterol molecules before it is cleaved and moved to the IMM (Artemenko et al., 2001; Hauet et al., 2005; Liu et al., 2006; Bose et al 2008).

Although StAR is necessary for the acute steroidogenic response, steroidogenesis still occurs in tissue where StAR is absent, such as the placenta and brain. However, the mechanism of StAR independent steroidogenesis is unknown and may involve the activity of other StAR like proteins such as MLN64 (metastatic lymph node clone 64) to promote cholesterol flux (Moog-Lutz et al., 1997; Miller and Auchus, 2011).

2.4 Enzymes involved in adrenal steroidogenesis

The synthesis of adrenal steroid hormones from cholesterol is catalyzed by two distinct groups of membrane bound enzymes, namely the cytochromes P450 (P450s) and the hydroxysteroid dehydrogenases (HSDs). The primary difference between these two groups is their catalytic mechanisms with the P450s containing a heme moiety necessary for catalysis whilst the HSDs do not (Payne and Hales, 2004; Miller and Auchus, 2011). The general characteristics of these two enzyme groups as well as their role in adrenal steroidogenesis will be discussed in the following sections.

2.4.1 The cytochromes P450

Cytochrome P450 is a term used to describe a large group of oxidative heme-containing enzymes present in all eukaryotic and in some prokaryotic organisms (Degtyarenko and Archakov, 1993; Meunier et al., 2004 and references therein). The P450s derive their name from their unique spectral properties. In their reduced state and in the presence of carbon monoxide these enzymes exhibit a distinct peak at 450 nm, hence the name P450 (Omura and Sato, 1962). The P450 enzymes are characterized by the presence of a single prosthetic group consisting of an iron (III) protoporphyrin-IX

ring covalently linked to the protein by the sulphur atom of a proximal Cys residue (Omura and Sato, 1964) (Fig 2.7). The heme iron of the protoporphyrin ring is penta- or hexacoordinated with the ring structure thus providing four ligands. The fifth ligand is provided by the sulphur atom of the cysteine residue whilst the sixth is believed to be water in the substrate free state. During the P450 catalytic cycle the sixth position becomes the site for dioxygen binding (Meunier et al., 2004; Denisov et al., 2005).

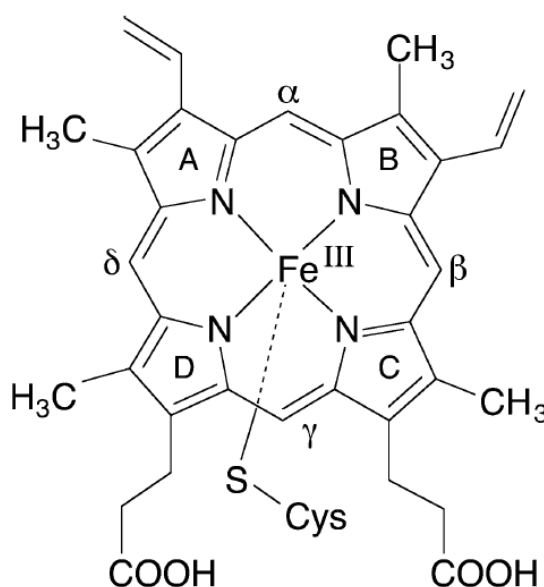
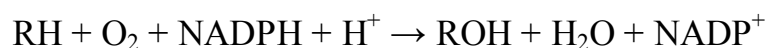


Figure 2.7 Cytochrome P450 prosthetic group. Reproduced from Meunier et al (2004).

P450 enzymes serve as strong oxidants and catalyze a variety of different oxidative reactions using molecular oxygen. During the catalytic cycle one oxygen atom is inserted into the substrate (RH) whilst the second is reduced to water through the utilization of two electrons provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH). Since the reaction only results in one oxygen atom being incorporated into the substrate, in the form of a hydroxyl group, P450s are often referred to as monooxygenases (Payne and Hales, 2004; Meunier et al., 2004; Miller and Auchus, 2011). The general reaction for P450 catalysis is given below.



The P450 catalytic cycle has been the subject of numerous studies resulting in the description of a general mechanism for substrate hydroxylation (Fig 2.8). Initially the enzyme is in the resting low-spin

(LS) ferric (Fe^{3+}) state with water coordinated as the sixth ligand (**1**). Upon substrate (RH) binding, the water molecule is displaced and excluded from the substrate binding pocket generating the high-spin (HS) substrate bound complex (**2**). The HS Fe^{3+} complex has a higher positive reduction potential which triggers the transfer of a single electron from the redox partner, reducing the complex to the ferrous (Fe^{2+}) state (**3**). The ferrous enzyme-substrate complex now binds molecular oxygen forming an oxygen-P450-substrate complex (**4**). The formation of this complex triggers the transfer of the second electron from the redox partner resulting in the formation of the Fe^{3+} -dioxo complex (**5a**). The Fe^{3+} -dioxo complex undergoes protonation to form a peroxo- Fe^{3+} intermediate (**5b**). The peroxo- Fe^{3+} intermediate undergoes a second protonation resulting in the splitting of the molecular oxygen. One of the oxygen atoms is transferred to water whilst the other remains bound to the ferric iron forming the reactive species (**6**). This species transfers the distal oxygen atom to the substrate (**7**). The hydroxylated substrate is released from the complex and replaced by water to regenerate the resting ferric state (**1**).

Apart from having multiple intermediate states, the P450 reaction cycle also contains three major abortive reactions. The first is the autoxidation of the oxy-ferrous complex (**4**) with the concomitant production of a superoxide and the return of the enzyme to the resting state (**2**). The second is a peroxide shunt where the coordinated hydroperoxide anion (**5b**) dissociates from the iron forming hydrogen peroxide and the return of the enzyme to the resting state. Lastly, an oxidase uncoupling can take place where the ferryl-oxo intermediate is oxidized to water instead of forming the hydroxylated substrate (**6**) (Gunsalus et al., 1975; Meunier et al., 2004; Bistolas et al., 2005; Denisov et al., 2005 and references therein).

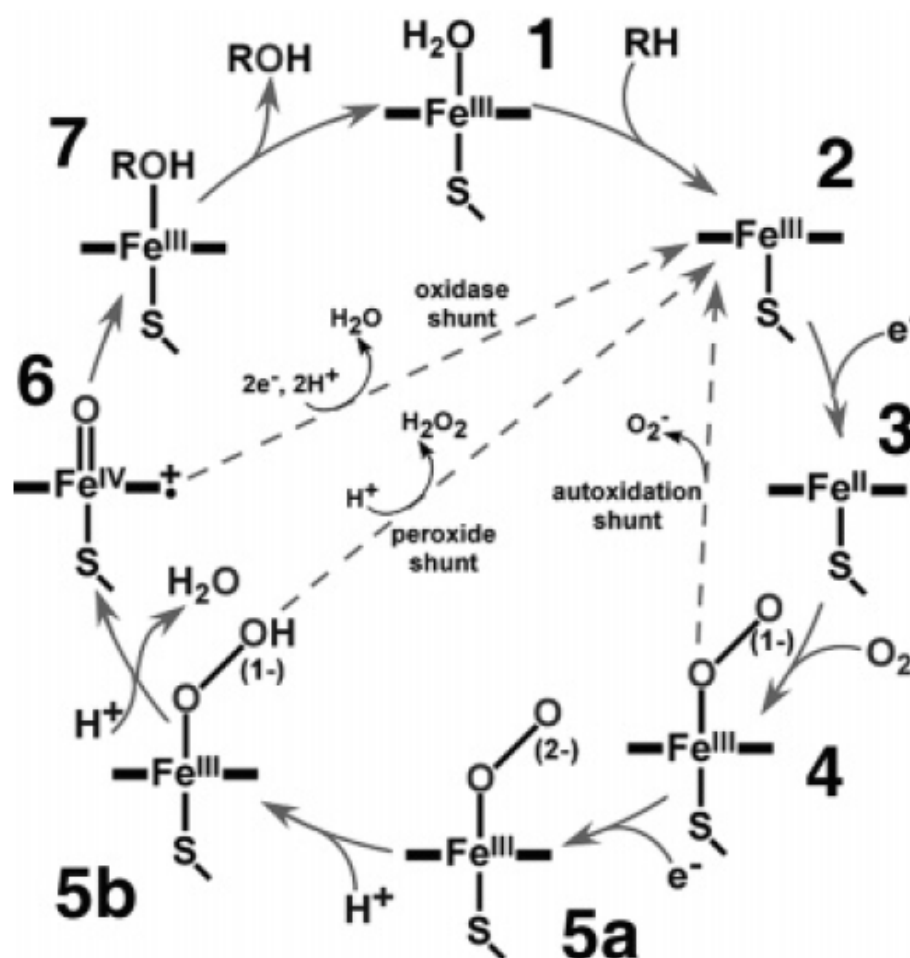


Figure 2.8 Reaction cycle of cytochromes P450. **RH** represents the substrate and **ROH** the hydroxylated product. Reproduced from Denisov et al (2005).

P450 enzymes are divided into ten different classes according to the mechanism by which they receive electrons as well as their intracellular localization (Hannemann et al., 2007 and references therein). In mammals, two classes are involved in the biosynthesis of steroid hormones, namely type-1 and type-2 targeted to the mitochondria and ER, respectively. Type-1 mitochondrial enzymes receive electrons from NADPH via two proteins. First, NADPH transfers the electron to a flavoprotein, adrenodoxin reductase, also known as ferredoxin reductase, which is subsequently transferred to an iron-sulphur protein, adrenodoxin, also known as ferredoxin. Finally, adrenodoxin transfers the electron to the substrate via the P450 heme center. Type-2 microsomal enzymes receive electrons from NADPH via a single 2-flavin moiety protein, cytochrome P450 oxidoreductase (POR). NADPH transfers the electron to flavinadenine dinucleotide (FAD) followed by transfer to flavinmononucleotide (FMN) and the P450 (Fig 2.9). Five P450s are involved in adrenal

steroidogenesis of which three are mitochondrial and two microsomal (Omura et al., 1966; Lu et al., 1969; Payne and Hales, 2004; Hannemann et al., 2007; Miller and Auchus, 2011).

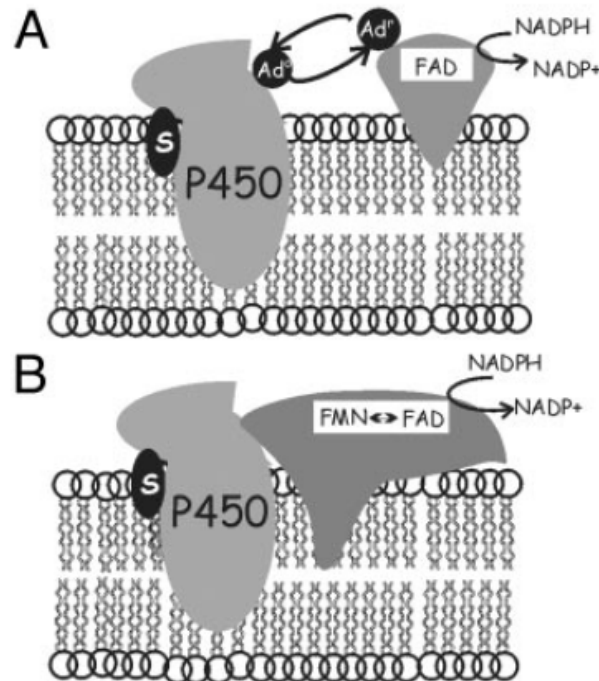


Figure 2.9 Schematic representation of mitochondrial and microsomal electron transfer. (A) Mitochondrial electron transfer, (FAD) flavinadenine dinucleotide; (Ad^{f}) adrenodoxin reductase; (Ad^{o}) adrenodoxin. (B) Microsomal electron transfer, (FMN) flavinmononucleotide; (S) substrate. Reproduced from Payne and Hales (2004).

2.4.2 The hydroxysteroid dehydrogenases

HSDs play a pivotal role in the biosynthesis and metabolism of steroid hormones. In steroidogenic tissue, HSDs catalyze the biosynthesis of mineralocorticoids, glucocorticoids, androgens and estrogens whilst in peripheral tissue they play a role in regulating gene expression by regulating the intracellular level of active steroid hormone (Samuels et al., 1951; Luu-The et al., 1989; Thomas et al., 1989; Penning, 1997; Payne and Hales, 2004; Simard et al., 2005).

One of the main differences between HSDs and P450s is that HSDs do not contain a heme group and catalyze reduction as well as oxidation reactions. HSDs utilize both nicotinamide adenine dinucleotide (NAD^+/NADH) and nicotinamide adenine dinucleotide phosphate ($\text{NADP}^+/\text{NADPH}$) as co-factor to either reduce or oxidize steroid substrates via a hydride transfer mechanism (Simard et al.,

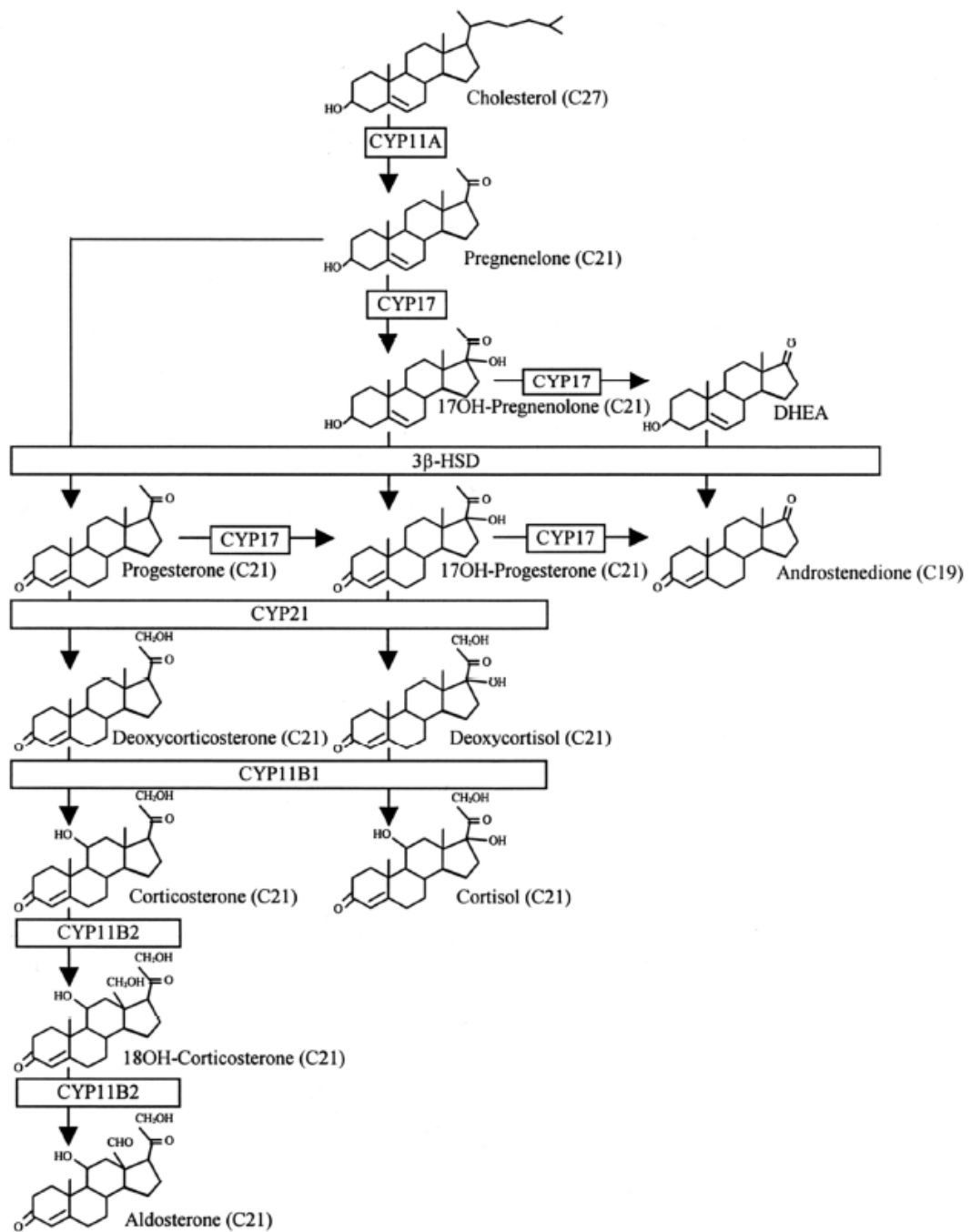
1996 and references therein). Another major difference is the number of isoforms or isozymes. Most P450 enzymes are products of a single gene with the steroidogenic reactions catalyzed by these enzymes being performed primarily by a single form of the enzyme. In contrast, HSDs are generally encoded by more than one gene resulting in more than one isoform catalyzing the same reaction. These isozymes are very different with variations in number, tissue distribution, subcellular localization, catalytic activity, co-factor and substrate specificity (Penning, 1997; Payne and Hales, 2004; Simard et al., 2005; Miller and Auchus, 2011 and references therein).

HSDs are divided into two groups according to their structure. These groups are the short-chain dehydrogenase/reductase (SDR) family and the aldo-keto reductase (ADR) family. However, based on their activities HSDs are also classified as dehydrogenases or reductases. The dehydrogenases utilize NAD^+ as co-factor to oxidize hydroxysteroids to ketosteroids whilst the reductases predominantly utilize NADPH to reduce ketosteroids to hydroxysteroids (Jörmvall et al., 1995; Penning, 1997; Kallberg et al., 2002; Miller and Auchus, 2011).

The only member of the HSD family that plays a role in adrenal steroidogenesis is 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3β HSD) (Penning, 1997 and references therein) which will be discussed in greater detail in the following chapter

2.5 The steroid hormone biosynthesis pathway

During adrenal steroidogenesis, six enzymes are involved in catalyzing the conversion of cholesterol to active steroid hormones — one is a member of the HSD family, namely 3β HSD, with the rest being P450s. The P450 enzymes involved in adrenal steroidogenesis include cytochrome P450 side-chain cleavage (CYP11A1), cytochrome P450 17α -hydroxylase/ $17,20$ lyase (CYP17), cytochrome P450 21 -hydroxylase (CYP21), cytochrome P450 11β -hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). In adrenocortical cells, steroidogenesis occurs in both the mitochondria and ER with the reactions catalyzed by CYP11A1, CYP11B1 and CYP11B2 occurring in the mitochondria whilst those catalyzed by 3β HSD, CYP17 and CYP21 take place in the ER (Fig 2.10).



Zona glomerulosa

Zona fasciculata

Zona reticularis

Figure 2.10 Overview of the individual reactions in the adrenal steroid hormone biosynthesis pathway. Reproduced from Slabbert (2003).

The first step in adrenal steroidogenesis occurs in the mitochondria where CYP11A1 catalyzes the conversion of cholesterol to pregnenolone (PREG). This conversion involves three distinct sequential reactions during which cholesterol undergoes 22-hydroxylation, 20-hydroxylation and finally oxidative scission of the C20-22 bond yielding PREG and isocaproaldehyde. PREG produced by this step, diffuses to the ER where it serves as substrate for either 3 β HSD or CYP17. If utilized by CYP17, PREG undergoes 17 α -hydroxylation to yield 17-hydroxypregnenolone (17-OHPREG) which in turn serves as substrate for the 17,20-lyase activity of CYP17 resulting in the cleavage of the C17-20 bond yielding DHEA. PREG, together with 17-OHPREG and DHEA, also serve as substrates for 3 β HSD that converts these Δ^5 steroids intermediates to their corresponding Δ^4 isoforms progesterone (PROG), 17-hydroxyprogesterone (17-OHPROG) and androstenedione (A4) through sequential dehydrogenase and isomerase reactions. CYP17 also catalyzes the hydroxylation of PROG to 17-OHPROG and its subsequent cleavage to A4. The PROG and 17-OHPROG produced by these steps further serve as substrates for CYP21, which catalyzes the hydroxylation of these steroids at C21 to yield deoxycorticosterone (DOC) and deoxycortisol, respectively. These steroids diffuse back to the mitochondria where CYP11B1 converts DOC to corticosterone (CORT) and deoxycortisol to cortisol. The final steps in adrenal steroidogenesis involve the conversion of corticosterone to 18-OH corticosterone and finally to aldosterone through the activity of CYP11B2 (Stewart, 2003; Payne and Hales, 2004; Miller and Auchus, 2011 and references therein). An overview of the individual reactions which makeup the adrenal steroid hormone biosynthesis pathway is shown in figure 2.10.

2.5.1 Factors that influence the steroidogenic output of the adrenal cortex

As previously mentioned in section 2.1, the zones of the adrenal cortex differ in their steroidogenic output. This can be attributed to the zone specific expression of specific steroidogenic enzymes. In the adult human adrenal the zona glomerulosa expresses CYP11A1, 3 β HSD, CYP21 and CYP11B2 with the notable exception of CYP17 and CYP11B1. Consequently, aldosterone is the primary steroid hormone produced by the zona glomerulosa. In contrast, the zona fasciculata expresses CYP11A1, 3 β HSD, CYP17 and CYP11B1 but does not express CYP11B2 and therefore primarily produces cortisol together with small amounts of DHEA and A4. Finally, the zona reticularis expresses CYP11A1, CYP17 but low levels of 3 β HSD, CYP21 and CYP11B1 leading to the production of DHEA, DHEA sulfate (DHEAS) and A4 together with trace amounts of cortisol (Stewart, 2003; Payne and Hales, 2004; Miller and Auchus, 2011 and references therein).

In addition to the zone specific expression of steroidogenic enzymes, the steroidogenic output of the adrenal cortex is influenced by the competition between key steroidogenic enzymes for steroid intermediates during steroidogenesis. Two enzymes that play a key role in the synthesis of all classes of adrenal steroid hormones are 3β HSD and CYP17. These enzymes compete for the Δ^5 steroid intermediates, PREG and 17-OHPREG (Fig 2.10), making the ratio of their enzyme activities and substrate specificities crucial in determining the flux of steroid intermediates through the steroidogenic pathways, which ultimately impacts adrenal steroidogenic output (Conley and Bird, 1997; Swart et al., 2003; Rainey and Nakamura, 2008; Conley et al., 2011).

This competition is further influenced by cytochrome-b₅ (Cyt-b₅). Cyt-b₅ is a small ubiquitous electron transfer hemoprotein well documented to selectively enhance the lyase activity of CYP17 with little effect on the hydroxylase activity. This augmentation of CYP17 lyase activity substantially alters the competition between 3β HSD and CYP17, resulting in a considerable increase in C19 (DHEA, A4) steroid production (Katagiri et al., 1982; Katagiri et al., 1995; Auchus et al., 1998).

Furthermore, developmental changes in the relative expression levels of 3β HSD, CYP17 and Cyt-b₅ in each cortical zone also influence this competition. Immunohistochemical analysis of the human adrenal cortex showed the expression of these proteins to vary between the three cortical zones during development. Suzuki et al (2000) showed that CYP17 expression in the zona fasciculata and reticularis increases from \approx 5 years of age and is highest in the zona reticularis whereas 3β HSD expression remained relatively constant in the zona fasciculata and glomerulosa but substantially decreased in the zona reticularis between the ages of 5-10 years. In contrast, Cyt-b₅ expression remained relatively constant in the zona fasciculata and glomerulosa, being the highest in the zona fasciculata, but substantially increased in the zona reticularis from the age of five (Fig 2.11). It is hypothesized that such developmental changes in the expression of these key steroidogenic enzymes results in an increase in DHEA and DHEAS production associated with the onset of adrenarche in humans and higher primates (Suzuki et al., 2000; Rainey et al., 2002; Havelock et al., 2004; Auchus and Rainey, 2004; Nguyen et al., 2009).

Therefore, it is clear that the steroidogenic output of the adrenal cortex is greatly influenced by the relative activity and substrate specificity of 3β HSD and CYP17 as well as the expression profile of these key enzymes together with Cyt-b₅ during adrenal development.

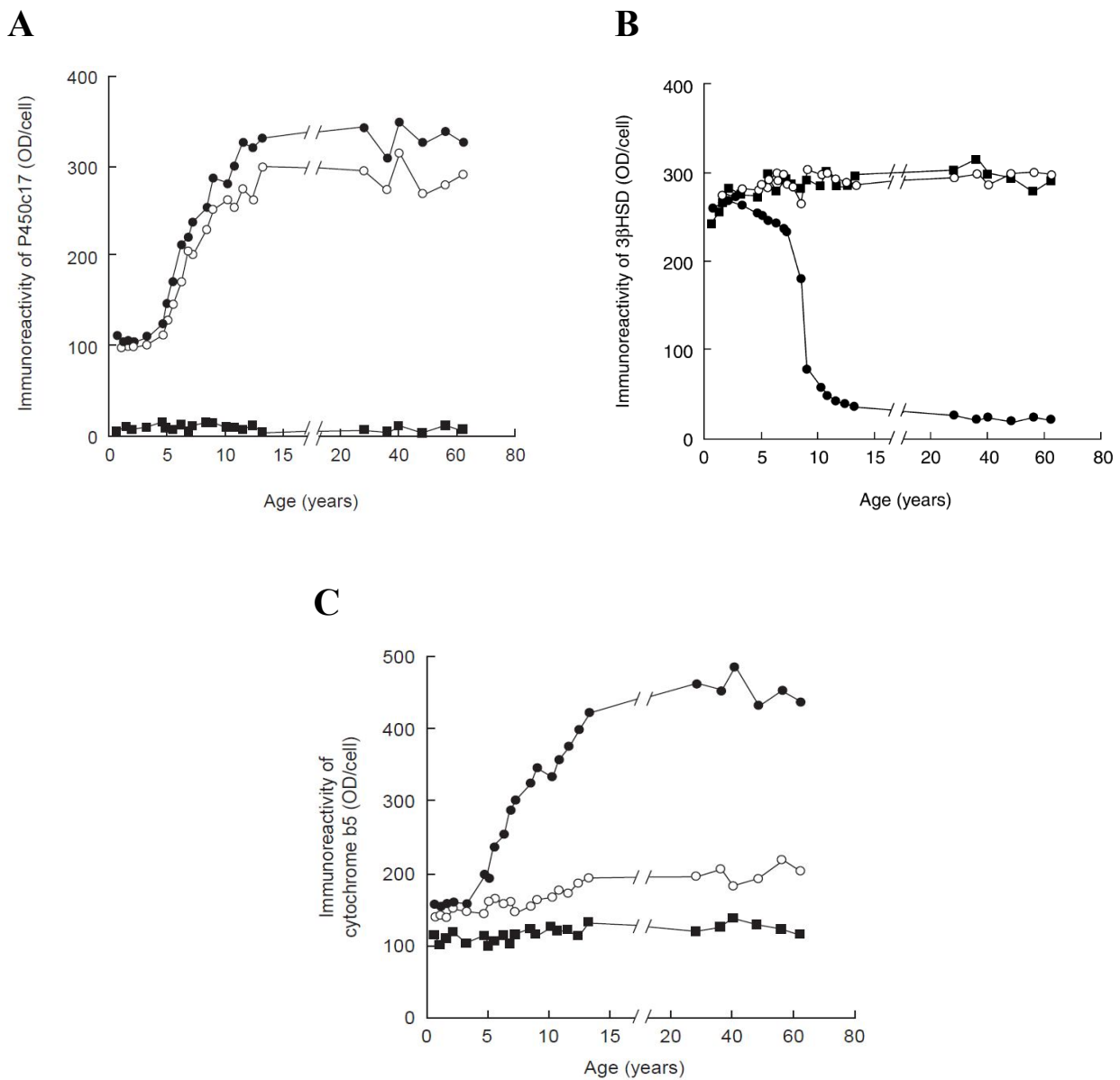


Figure 2.11 Age related changes in immunoreactivity of CYP17 (A), 3βHSD (B) and Cyt-b₅ (C) in the human adrenal cortex. Zona reticularis (●), zona fasciculata (○) and zona glomerulosa (■). Reproduced from Suzuki et al (2000).

2.6 Summary

From the preceding discussion it is clear that steroid hormones play an essential role in mediating a variety physiological processes and that abnormal production of these hormones may result in a number of clinical disorders, as illustrated for cortisol biosynthesis in the South African Angora goat.

In mammals, these hormones are primarily synthesized in the adrenal cortex from cholesterol through a number of enzyme catalyzed reactions, collectively known as the steroid hormone biosynthesis pathway (Payne and Hales, 2004; Miller and Auchus, 2011). One of the most important enzymes in the context of steroid hormone production is 3 β HSD as it plays a key role in the synthesis of all classes of adrenocortical hormones (Simard et al., 2005). Furthermore, 3 β HSD activity also determines the flux of steroid intermediates through the various steroidogenic pathways due to its competition with CYP17 for Δ^5 steroid intermediates (Conley and Bird, 1997; Swart et al., 2003; Rainey and Nakamura, 2008; Conley et al., 2011). This competition is further influenced by Cyt-b₅ and the relative expression level of 3 β HSD, CYP17 and Cyt-b₅ in the respective zones of the adrenal cortex.

Thus, the steroidogenic output of the adrenal may be greatly influenced by the relative activity, substrate specificity and expression level of this key steroidogenic enzyme. The following chapter will therefore specifically focus on the physiological importance of 3 β HSD as well as its structure, catalytic mechanism and tissue specific expression.

CHAPTER 3

3 β -HYDROXYSTEROID DEHYDROGENASE/ Δ^5 - Δ^4 ISOMERASE

3.1 Introduction

3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β HSD) is a dimeric, membrane-bound enzyme essential in the production of all classes of steroid hormones. This enzyme is usually present in multiple isoforms within the same species, with each isoform being the product of a distinct gene. These isoforms are expressed in a tissue specific manner and vary in sub-cellular localization, catalytic activity as well as substrate and co-factor specificity (Labrie et al., 1992; Payne and Hales, 2004; Simard et al., 2005). In humans, two 3 β HSD isoforms have been characterized, namely 3 β HSD type I and II (3 β HSD I, II) (Luu-The et al., 1989; Rheaume et al., 1991). A number of other 3 β HSD isoforms have also been characterized from a variety of different species, including mouse, rat and hamster (Zhao et al., 1991; Bain et al., 1991; Rogerson et al., 1995). These exhibit a high degree of homology in their amino acid sequences and are classified into two functional groups depending on their catalytic function. The first group functions as classic dehydrogenase/isomerases utilizing NAD⁺/NADH as co-factor whereas the second group functions as 3-ketosteroid reductases utilizing NADP⁺/NADPH as co-factor (Clarke et al., 1993; Penning, 1997; Payne and Hales, 2004; Simard et al., 2005). During adrenal steroidogenesis, 3 β HSD functions as a dehydrogenase/isomerase, catalyzing the conversion Δ^5 steroid intermediates to their corresponding Δ^4 products. This chapter will discuss the remarkable diversity within the 3 β HSDs as a group of enzymes, with specific focus on the adrenal isoform, to illustrate the vital role this enzyme plays in maintaining normal physiological processes through the synthesis and metabolism of various steroid hormones.

3.2 Catalytic function

3 β HSD is a bi-functional enzyme converting Δ^5 steroid intermediates to their corresponding Δ^4 products in various steroidogenic tissues. During adrenal steroidogenesis 3 β HSD catalyzes the conversion of PREG, 17OHPREG and DHEA to PROG, 17-OHPROG and A4, respectively (Fig 3.1). Therefore, 3 β HSD activity is required for the synthesis of all classes of steroid hormones as PROG, 17-

OHPROG and A4 serve as precursors for the synthesis of mineralocorticoids, glucocorticoids, androgens and estrogens.

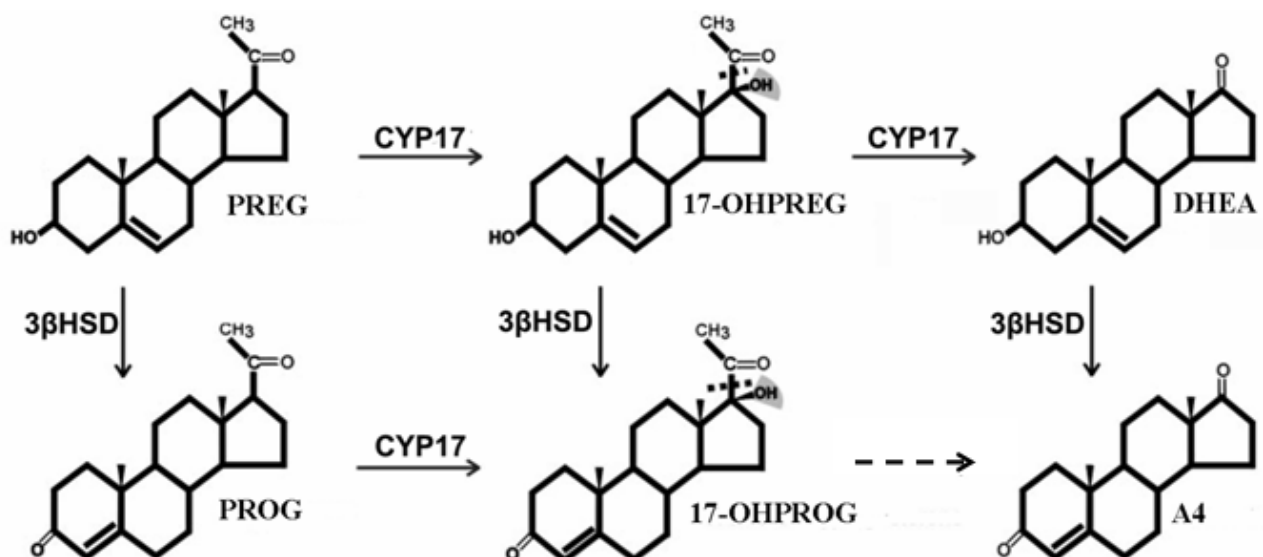


Figure 3.1 Steroidogenic reactions catalyzed by 3βHSD during adrenal steroidogenesis. The diagram illustrates the competition between 3βHSD and CYP17 for the Δ^5 steroid intermediates, PREG and 17-OHPREG.

The conversion of Δ^5 -hydroxysteroids to Δ^4 -ketosteroids, by 3βHSD occurs via sequential dehydrogenase and isomerase reactions during which the hydroxyl group on carbon 3 is converted to a keto group followed by the isomerization of the double bond from the B-ring (Δ^5) to the A-ring (Δ^4) (Fig 3.2) (Thomas et al., 1989; Lachance et al., 1990; Lorence et al., 1990). In the first and rate-limiting dehydrogenase reaction, NAD^+ is reduced to NADH with the formation of a Δ^{5-3} keto steroid intermediate which, together with NADH, remains bound to the enzyme. Stopped-flow spectroscopy studies have shown that the NADH subsequently induces time-dependant conformational changes in the enzyme resulting in the isomerization of the intermediate to form the corresponding Δ^4 steroid product, after which the product is released (Fig 3.2) (Thomas et al., 1989, 1995, 2003). According to the model proposed by Thomas et al. (1995), the dehydrogenase and isomerase domains of the enzyme are linked by a separate shared coenzyme domain that functions as both the binding site for NAD^+ during the dehydrogenase reaction and for the reduced NADH, with the latter subsequently acting as an allosteric activator of the isomerase reaction.

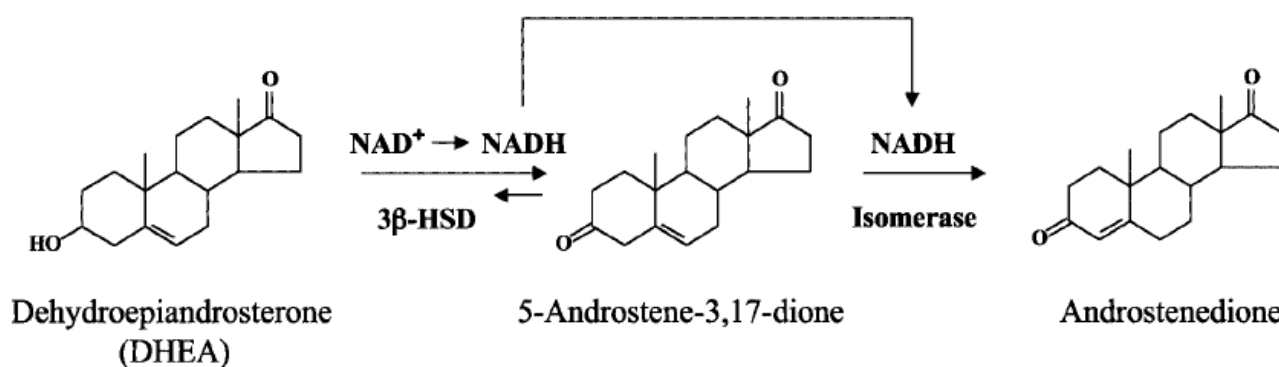


Figure 3.2 Sequential dehydrogenase and isomerase reactions during the conversion of DHEA to androstenedione via a Δ^{5-3} keto intermediate, 5-androstene-3,17-dione. Reproduced from Thomas et al (2005).

3.3 Structure-function relationships

3 β HSD is a membrane bound enzyme localized to the endoplasmic reticulum and mitochondrial membrane in steroidogenic tissues (Beyer and Samuels, 1956; Chapman and Sauer, 1979; Thomas et al., 1989; Cherradi et al., 1995; Simard et al., 2005). Due to the inherent difficulty of crystallizing relatively hydrophobic membrane bound proteins, no crystal structure is currently available describing the 3-D structure of 3 β HSD. To date, the majority of data describing structure-function relationships for 3 β HSD have been obtained through site-directed mutagenesis. More recently, however, structure-function relationships within 3 β HSD have also been investigated using homology modeling, a technique used to predict the 3-D structure of a protein based on the observation that proteins with similar, but not identical, amino acid sequences tend to adopt similar 3-D structures (Chothia and Lesk, 1986). The use of this technique has enabled the development of hypotheses regarding various structure-function relationships, including those involved in co-factor binding and dehydrogenase and isomerase activity, which could be validated by site-directed mutagenesis.

Initial studies investigating 3 β HSD membrane-binding identified two putative membrane-binding domains in the primary structure of 3 β HSD using hydrophobicity analyses (Simard et al., 1996). In human 3 β HSD the first of these hydrophobic segments is located in the NH₂-terminal region between residues 72 and 89 whilst the second is located in the COOH-terminal region between residues 283 and 310. Deletion of the 283-310 region in human type I 3 β HSD produced a cytosolic form of the enzyme that retains both dehydrogenase and isomerase activity comparable to that of the wild type enzyme. This suggests that this region is not essential for enzyme activity but plays a crucial role in

membrane-binding. In contrast, deletion of the 72-89 region produced a mutant protein that is distributed between the endoplasmic reticulum, mitochondria and cytosol with 28% of the protein being retained in the membrane. Furthermore, deletion of this region also resulted in an 8 fold loss in both dehydrogenase and isomerase activities, compared to the wild type enzyme, suggesting that this region not only contributes to membrane association but possibly enzyme function as well (Thomas et al., 1999; 2001).

Isoforms of 3 β HSD exhibit strict co-factor specificity with those acting as dehydrogenase/isomerases utilizing NAD(H) as co-factor whilst those acting as reductases utilize NADP(H) (Abbaszade et al., 1995; Thomas et al., 2003; Miller and Auchus, 2011). As a member of the short-chain dehydrogenase/reductase (SDR) family of enzymes, 3 β HSD is characterized by an NH₂-terminal Rossmann fold coenzyme domain. This domain is highly conserved and consists of a β - α - β - β - α - β motif that contains a Gly-rich region, Gly-X-X-Gly-X-X-Gly, between the first β -strand and α -helix. This Gly-rich region provides a hydrophobic pocket for the adenosine monophosphate (AMP) moiety of the co-factor, allowing for close association between the enzyme and the co-factor (Jörnvall et al., 1995; Simard et al., 2005; Kavanagh et al., 2008 and references therein).

Wierenga and co-workers were the first to suggest that the region between amino acid residues 36 and 38, located in the β - α - β fold of the co-factor binding domain of NAD⁺-requiring dehydrogenases, could play a role in co-factor specificity. They proposed that an acidic residue at position 36 exhibits negative interference with the 2'-phosphate group of NADP(H), and as such prevents the dehydrogenases from utilizing NADP(H) as co-factor (Wierenga and Hol, 1983; Wierenga et al., 1986). Subsequent studies investigating co-factor specificity between different murine 3 β HSD isoforms found that Asp³⁶ was essential for the NAD⁺-mediated dehydrogenase activity of mouse 3 β HSD I. Substitution of Asp³⁶ with a Phe residue shifted co-factor specificity to NADP(H). Mouse 3 β HSD IV and V for which NADP(H) is the preferred co-factor, both contain a Phe residue at position 36 (Abbaszade et al., 1995).

More recently, a homology model of human type I 3 β HSD was constructed which illustrated the importance of residues 36 and 37 in co-factor specificity. In these studies, a Asp36Ala/Lys37Arg mutation in human 3 β HSD I shifted co-factor specificity from NAD⁺ to NADP⁺ for both the dehydrogenase and isomerase activities of the enzyme. Using their homology model, the authors concluded that the strict NAD⁺ specificity of the enzyme may be due to hydrogen bonding between the carboxylate R-group of Asp³⁶ and the 2', 3'-hydroxyl groups of the adenosyl ribose group of NAD⁺ whilst repelling the 2'-phosphate group of NADP⁺, preventing its utilization. However, in the Asp36Ala/Lys37Arg mutant the nitrogen atoms of the guanidinium group of Arg³⁷ neutralize the 2'-

phosphate group of NADP⁺ through hydrogen bonding, thus allowing the utilization of NADP⁺ as co-factor (Fig 3.3). In addition, since both the dehydrogenase and isomerase activities were equally affected by these substitutions, the authors concluded that both activities share a single co-enzyme domain (Thomas et al., 2003).

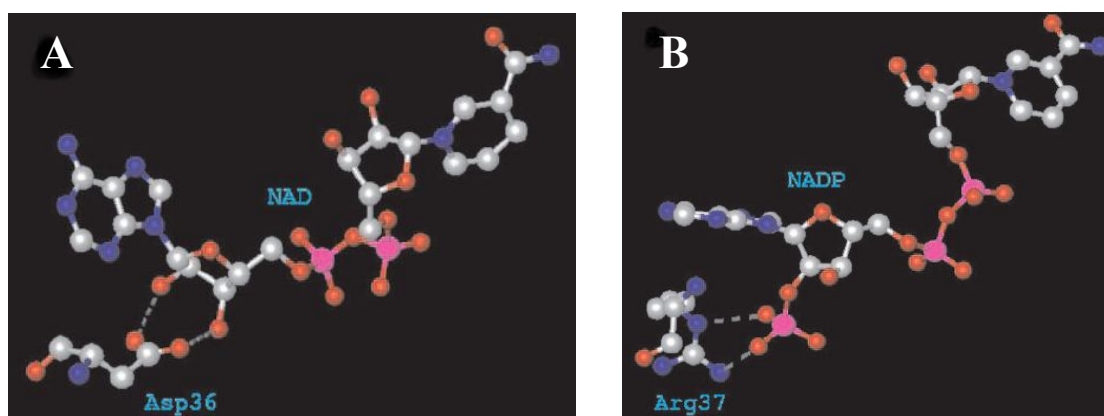


Figure 3.3 Interactions of Asp³⁶ with NAD⁺ (A) and Arg³⁷ with NADP⁺ (B) in human type I 3βHSD based on a homology model. Hydrogen bonds are shown in both panels by grey dotted lines. In both panels, Carbon atoms are grey; Oxygen atoms, orange; Nitrogen atoms, purple and Phosphorus atoms, lavender. Reproduced from Thomas et al (2003).

Members of the SDR family typically contain a single catalytic Tyr-X-X-X-Lys motif (Chen et al., 1993; Jörmvall et al., 1995; Tanabe et al., 1998), usually associated with dehydrogenase activity (Thomas et al., 2002). However, the 3βHSDs all contain two potential catalytic motifs (Luu-The et al., 1989; Simard et al., 2005). In the primary structure of human 3βHSD these motifs are; Tyr¹⁵⁴-X-X-X-Lys¹⁵⁸ and Tyr²⁶⁹-X-X-X-Lys²⁷³. Studies investigating 3βHSD substrate binding initially identified two tryptic peptides in the primary structure of human 3βHSD I which contained these catalytic motifs. The first peptide comprised of amino acid residues Gly²⁵¹ to Lys²⁷⁴ and contained the Tyr²⁶⁹-X-X-X-Lys²⁷³ catalytic motif whilst the second comprised of amino acid residues Glu¹³⁵ to Lys¹⁵⁸ and contained the Tyr¹⁵⁴-X-X-X-Lys¹⁵⁸ motif. Site-directed mutagenesis studies showed Tyr²⁵³ to play a critical role in isomerase activity, placing the Tyr²⁶⁹-X-X-X-Lys²⁷³ motif in the isomerase domain (Fig 3.4) (Thomas et al., 1993; 1997; 1998).

Subsequent studies therefore investigated whether the Tyr¹⁵⁴-X-X-X-Lys¹⁵⁸ motif participates in the dehydrogenase reaction by comparing the primary structures and substrate kinetics of human 3βHSD type I and II. The dehydrogenase activities of these isoforms differ with the type I enzyme

exhibiting a ≈ 14 -fold higher affinity for the substrate. Alignment of the primary sequences of human type I and II 3β HSD showed both isoforms to possess analogous Tyr¹⁵⁴-X-X-X-Lys¹⁵⁸ motifs which differ by a single amino acid residue: Tyr¹⁵⁶ (type I) and His¹⁵⁶ (type II). Using site-directed mutagenesis, a His156Tyr mutation in type I was shown to shift the substrate kinetics to that of the type II enzyme. These studies illustrated that His¹⁵⁶ plays an important role in the binding of steroid substrate and suggested a catalytic role for Tyr¹⁵⁴ and Lys¹⁵⁸ during the dehydrogenase reaction (Fig 3.4) (Thomas et al., 2002). In addition, more recent studies have also implicated Gln¹⁰⁵, Gln²⁴⁰, Asp⁶¹ and Arg¹⁹⁵ as contributing towards the higher affinity of the type I enzyme for substrates, co-factors and inhibitors compared to the type II enzyme (Thomas et al., 2005; 2010).

As mentioned above, the predicted 3-D structure currently available for 3β HSD was obtained by site-directed mutagenesis and homology modeling, and can only be verified once the crystal structure for 3β HSD has been solved.

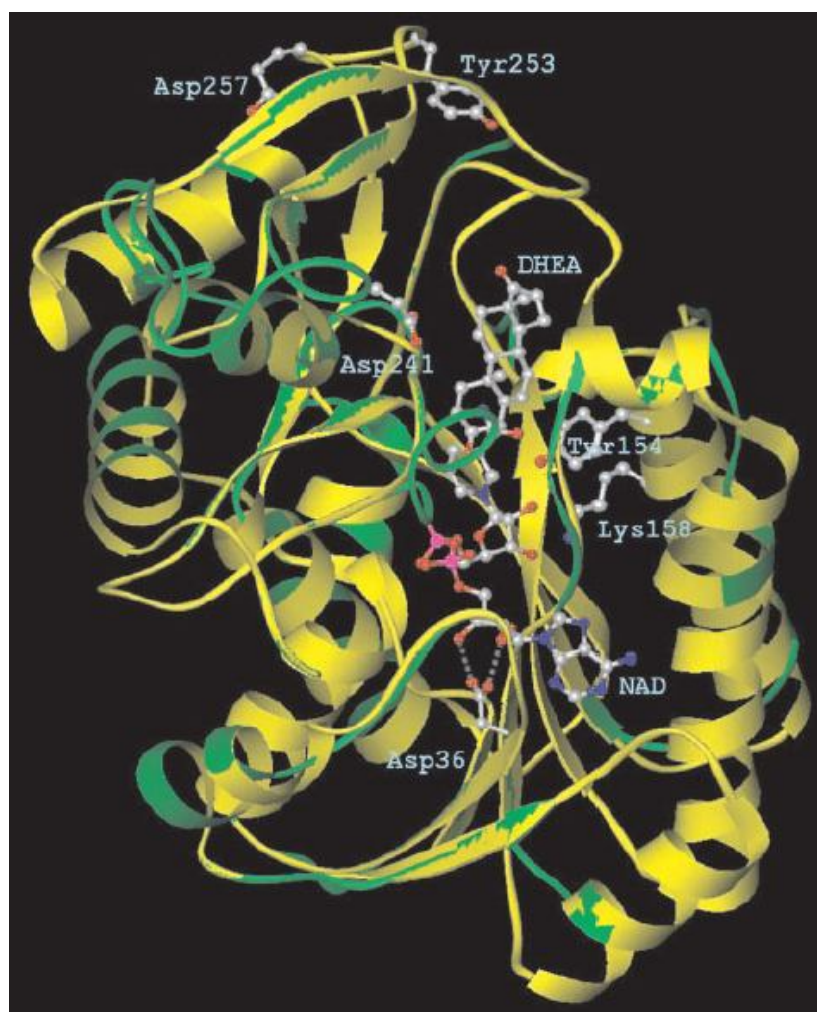


Figure 3.4 Homology model of human type I 3βHSD. Human type I 3βHSD (green) aligned with UDP-galactose-4-epimerase (yellow) shows the putative 3-D structure in the dehydrogenase conformation and includes the NAD⁺ co-factor and the steroid substrate DHEA. Grey dotted lines indicate Asp³⁶ hydrogen bonds with the 2', 3'-hydroxyl groups of the NAD⁺ moiety. Tyr¹⁵⁴ and Lys¹⁵⁸ implicated in dehydrogenase activity and Tyr²⁵³ and Asp²⁵⁷ implicated in isomerase activity are also shown. The carbon atoms are grey; Oxygen atoms, orange; Nitrogen atoms, purple and Phosphorus atoms, lavender. Reproduced from Thomas et al (2003).

3.4 Isoforms of 3βHSD

As mentioned in section 2.4.2 3βHSDs are generally encoded by more than one gene resulting in more than one isoform catalyzing the same reaction. These isoforms were found to be expressed in a tissue- and developmental specific manner, with their catalytic activity and function also varying (Labrie et al., 1992; Simard et al., 1996; Penning, 1997; Payne and Hales, 2004). In addition to those

identified in humans, multiple 3 β HSD isoforms have also been cloned from a number of other species. These include: mouse (Bain et al., 1991), rat (Zhao et al., 1991), bovine (Zhao et al., 1989), macaque (Simard et al., 1991a), chicken (Nakabayashi et al., 1995) and horse (Hasegawa et al., 1998). The best characterized 3 β HSD isoforms are those from the mouse, human and rat. The following section will discuss the characteristics of the 3 β HSD isoforms present in these three species to illustrate the remarkable diversity of activity and tissue specific expression within the 3 β HSD group of enzymes.

3.4.1 Human isoforms

As previously mentioned, two 3 β HSD isoforms have been characterized in humans, namely 3 β HSD type I and II. The first cDNA sequence to be characterized was that of the type I enzyme following its purification from human placenta (Luu-The et al., 1989, 1990; Lorence et al., 1990; Nickson et al., 1991). The second isoform, chronologically designated as type II, was isolated from a human adrenal cDNA library (Rheume et al., 1991). The 3 β HSD type I gene (*HSD3B1*) encodes a 372 amino acid protein that is predominantly expressed in the placenta, skin and breast tissue as well as other peripheral tissues such as the prostate, liver, brain and tumor tissues (Dumont et al., 1992; Gingras and Simard, 1999; Gingras et al., 1999; Simard et al., 2005; Miller and Auchus, 2011). In contrast, the 3 β HSD type II gene (*HSD3B2*) encodes a 372 amino acid protein that is almost exclusively expressed in the adrenals, ovaries and testis (Lachance et al., 1991). Furthermore, in addition to the tissue specific expression of these isoforms, 3 β HSD expression also varies in a developmental specific manner. For example, in the adrenal the expression of the type II enzyme varies between the different zones depending on developmental stage. Immunohistochemical analysis of the level of 3 β HSD II present in the human adrenal cortex revealed considerable age-related changes in immunoreactivity in the different cortical zones. Between the ages of 0 -5 years, the level of 3 β HSD is similar in all three zones. In contrast, from the age of 5 years the level of 3 β HSD present in the zona reticularis decreases considerably whilst remaining relatively constant in the zona fasciculata and glomerulosa (Gell et al., 1998; Dardis et al., 1999; Suzuki et al., 2000).

In addition to the differences in expression profile, these isoforms also exhibit significant differences in catalytic activity. Although these enzymes share 93.5% identity in their primary structure they differ significantly in their affinity for both the dehydrogenase and isomerase substrates, as illustrated in Table 3.1.

Table 3.1. Comparison of kinetic parameters between human 3 β HSD type I and II. Adapted from Thomas et al (2005).

Purified 3 β HSD	DHEA		NAD ⁺		5-Androstene-3,17-dione		NADH	
	K_m	K_{cat}	K_m	K_{cat}	K_m	K_{cat}	K_m	K_{cat}
	μM	min^{-1}	μM	min^{-1}	μM	min^{-1}	μM	min^{-1}
Type I	3.7	3.3	34.1	3.5	27.9	50.2	4.6	45
Type II	47.3	7.1	86.3	7.1	88.4	81.5	12.6	99.1

Experimental data shows that the type I enzyme exhibits significantly higher substrate affinities compared to the type II enzyme (Table 3.1) (Lachance et al., 1990; Rheaume et al., 1991; Thomas et al., 2001; Simard et al., 2005). It has been suggested that the higher affinity of the type I enzyme, predominantly expressed in peripheral tissues, could facilitate the formation of steroid hormones in these tissues where the concentration of steroid substrates are usually low (Simard et al., 2005). However, the exact reason for the differences in substrate affinity exhibited by different 3 β HSD isoforms and how these differences influence steroid biosynthesis and metabolism in various tissues is still unclear.

The structures of both human 3 β HSD genes have been determined. Both genes are ≈ 7.8 kb in length and consist of four exons and three introns with the exons sharing 77.4, 91.8, 94.5 and 91% identity (Lorence et al., 1990b; Lachance et al., 1991). These genes are localized to chromosome 1p13.1 at a distance of 1-2 cM from the D1Z5 centromeric marker. Initial studies investigating the *HSD3B1* and *HSD3B2* genes identified three additional pseudogenes and suggested that the human 3 β HSD gene family exists as a tandem cluster of related genes (Berude et al., 1989; Morissette et al., 1995). More recent studies have identified two additional pseudogenes which are closely related to the *HSD3B1* and *HSD3B2* genes but contain no corresponding open reading frames. These studies showed that even though mRNA is expressed from these pseudogenes the presence of altered splice sites disrupt the reading frames, preventing the expression of these genes. Furthermore, the *HSD3B1* and *HSD3B2* genes have also been shown to be separated by a 100 kb segment, preventing these genes from sharing common promoter elements (Fig 3.5) (McBride et al., 1999; Simard et al., 2005).

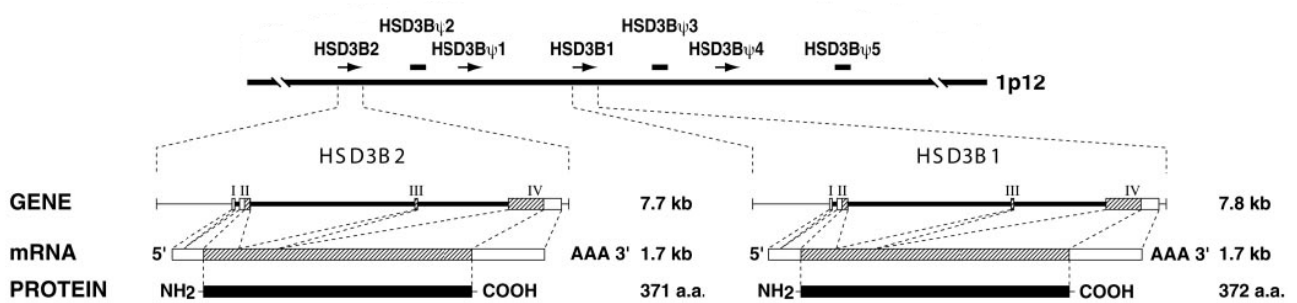


Figure 3.5 Human 3β HSD genes and pseudogenese. The locations of the two expressed genes, *HSD3B1* and *HSD3B2*, relative to the five pseudogenes (*HSD3Bψ1-5*) are shown in the uppermost diagram. The orientation of the genes are indicated by arrows which point in the direction of the stop codon. The structure of both the type I and type II genes, their mRNA species as well as their corresponding protein products are shown in the bottom two diagrams. The four Exons in each 7.7/7.8 kb gene are presented in boxes containing hatched lines representing the coding regions. The three introns in each gene are illustrated by black bold lines. Reproduced from Simard et al (2005).

3.4.2 Mouse isoforms

The mouse 3β HSD gene family represents the largest group of 3β HSD isoforms. To date, six distinct 3β HSD cDNA sequences have been isolated and characterized (Bain et al., 1991; Clarke et al., 1993a, b; Abbaszade et al., 1995, 1997). These exhibit a high degree of sequence identity and all contain a 1122 bp open reading frame encoding a 373 amino acid protein (Abbaszade et al., 1997; Peng et al., 2002). Based on their function, the isoforms are grouped into two functional groups. The first group, comprising 3β HSD type I, III, VI and possibly type II, functions as NAD^+ -dependant dehydrogenase/isomerases that are essential for the synthesis of active steroid hormones. The second group, comprised of 3β HSD type IV and V, functions as NADPH-dependant 3-ketosteroid reductases involved in the inactivation of active steroid hormones (Abbaszade et al., 1995, 1997; Peng et al., 2002; Payne and Hales, 2004).

Like the isoforms from other species, mouse 3β HSD isoforms are also expressed in a tissue- and developmental specific manner. In the adult mouse, 3β HSD I is the major isoform expressed in the adrenals and gonads and is therefore the primary isoform responsible for the biosynthesis of steroid hormones in these tissues. The type II and III enzymes are expressed in the liver and kidney, with type III being the predominant isoform expressed in the adult liver (Bain et al., 1991; Abbaszade et al., 1995). The predominant isoform expressed in the adult mouse male and female kidney is 3β HSD IV.

Its expression has been found to be localized only to the cortex, with the highest expression occurring in the convoluted tubules (Clarke et al., 1993b). The type V enzyme is expressed in the liver of male mice only (Abbaszade et al., 1995; Park et al., 1996). 3 β HSD VI, the ortholog of human 3 β HSD I, is expressed in the skin and testis of adult mice as well as during pregnancy in deciduas and giant trophoblast cells (Abbaszade et al., 1997; Arensburg et al., 1999; Baker et al., 1999).

Similar to human 3 β HSD genes, mouse 3 β HSD genes also consist of four exons with the size of each gene varying between 6 and 11 kb depending on the size of the introns. The first exon contains the 5' untranslated sequence whilst the second contains the translation start site. The third exon is short with the majority of the translated sequence being present in the forth exon. Analysis of the mouse 3 β HSD genes identified seven genes located in a 400 kb fragment on mouse chromosome 3, suggesting that these genes exists as a tandem cluster of related genes (Clarke et al., 1996; Peng et al., 2002; Simard et al., 2005).

Table 3.2 Functional classification and tissue specific expression of human, mouse and rat 3 β HSD isoforms. Adapted from Peng et al (2002).

Isoforms	Tissue	References
<i>NAD⁺-dependant dehydrogenase/isomerases</i>		
Human II	Adrenals, gonads	Lachance et al., 1991
Mouse I	Adrenals, gonads, fetal and neonatal liver	Bain et al., 1991; Park et al., 1996
Rat I	Adrenals, gonads, kidneys	Zhao et al., 1991; Simard et al., 1993a
Rat II	Adrenals, gonads, fat	Zhao et al., 1991; Simard et al., 1993a
Human I	Placenta, skin, mammary glands	Lorence et al., 1990; Rheaume et al., 1991
Rat IV	Placenta, skin	Simard et al., 1993a
Mouse VI	Maternal decidua, giant tropholasts, skin, testis	Abbaszade et al., 1997
Mouse II	Kidneys, liver	Bain et al., 1991
Mouse III	Liver, kidneys	Bain et al., 1991; Abbaszade et al., 1995
<i>NADPH-dependant 3-ketosteroid reductases</i>		
Rat III	Male liver	Zhao et al., 1990 ; de Launoit et al., 1992
Mouse V	Male liver	Abbaszade et al., 1995
Mouse IV	Kidneys	Clarke et al., 1993

3.4.3 Rat isoforms

The cDNA structures of four members of the rat 3 β HSD family have been characterized and all encode a 372 amino acid protein. The type I, II and IV enzymes function as NAD⁺-dependant dehydrogenase/isomerases whilst the type III enzyme functions as a NADPH-dependant 3-ketosteroid reductase (Zhao et al., 1990, 1991; Labrie et al., 1992; Simard et al., 1993a; Simard et al., 1996). The primary structures of the type I and II enzymes share 93.8% sequence identity and are expressed in the adrenals, gonads, kidney, placenta, adipose tissue and uterus. In contrast, the type III enzyme exhibits only 80% identity with the type I and II enzymes and is expressed exclusively in the liver of male rats. This sexual dimorphic expression has been shown to be the result of pituitary hormone induced gene repression in the female rat liver preventing the expression of the type III enzyme in female rats (Zhao et al., 1990; Couet et al., 1992). The type IV enzyme is the predominant isoform expressed in the placenta and skin and has also been detected in the ovary and to a lesser degree in the adrenals (Simard et al., 1993a). Studies investigating the activity of the type I, II and IV enzymes found that the type I and IV enzymes exhibit similar activities towards PREG and DHEA. In contrast, the type II enzyme exhibits a much lower activity towards these substrates which the authors attributed to amino acid residue differences in the putative membrane spanning domain between residues 75 and 91 (Simard et al., 1991b, 1993a).

3.5 Sub-cellular localization

In steroidogenic tissue, the majority of steroidogenic enzymes involved in steroidogenesis are localized to the smooth ER with the exception of CYP11A1, CYP11B1 and CYP11B2. However, 3 β HSDs are unique in that they show dual sub-cellular localization with the degree of distribution between the ER membrane and mitochondrial membrane varying between different tissues and species (Payne and Hales, 2004; Simard et al., 2005; Miller and Auchus, 2011).

Initial studies investigating the intracellular localization of 3 β HSD in bovine adrenal cortex tissue revealed 3 β HSD activity to be distributed between the microsomal (39%), mitochondrial (14%) and nuclear (26%) fractions. It was found that the activity in the nuclear fraction could be eliminated by repeated washing steps but that this technique had no effect on the mitochondrial activity which could only be removed through detergent solubilization. However, despite these observations, the authors concluded that the 3 β HSD activity observed in the mitochondrial fraction was due to microsomal contamination leading to the general acceptance that 3 β HSD is a constituent of the smooth ER only

(Beyer and Samuels, 1956; Chapman and Sauer, 1979). Following this initial study a controversy developed over the possible localization of 3 β HSD to the mitochondria as several studies reported 3 β HSD activity in mitochondria of ovaries (Dimino and Campbell, 1976), testes (Sulimovici et al., 1973), human placenta (Ferre et al., 1975) and rat adrenal cortex (Kream and Sauer, 1976). These results were disputed by other investigators who attributed the presence of 3 β HSD in the mitochondria to either microsomal contamination (Moustafa and Koritz, 1975; Caffrey et al., 1979) or an enzyme redistribution artifact resulting from homogenization (Cowan et al., 1974). Nevertheless, results from a number of studies in various different species have now shown that mitochondrial fractions from most steroidogenic tissues do indeed contain 3 β HSD activity (Hiura et al., 1981; Cherradi et al., 1993, 1994, 1997; Pelletier et al., 2001). The development of antibodies against 3 β HSD has also further verified the dual-localization of 3 β HSD to the ER and mitochondria (Perry et al., 1991).

The sub-cellular distribution of 3 β HSD has also been shown to differ between species and between different tissues. Immunohistochemical studies in bovine, mouse and rat adrenocortical tissue revealed 3 β HSD to be localized solely to the microsomal (ER) fraction of bovine and mouse adrenal glands whilst exhibiting dual localization to the microsomal and inner mitochondrial membrane fractions of rat adrenal glands (Perry et al., 1991). Furthermore, studies in the rat adrenal cortex and gonads have also demonstrated that the degree of 3 β HSD distribution between the ER and mitochondria also varies between different tissues. In the ovary and adrenal cortex, immunohistochemical analysis revealed 3 β HSD to be localized mostly to the ER and some to the mitochondria whilst in the testis 3 β HSD was observed exclusively in the mitochondria of the leydig cells (Pelletier et al., 2001; Payne and Hales, 2004).

At present, the relevance of dual localization in the ER and mitochondria is unclear. It has, however, been hypothesized that it may serve as a means to regulate the steroidogenic output of the cell through substrate availability. According to this hypothesis, 3 β HSD localized to the inner mitochondrial membrane would have less access to cytosolic steroid substrates such as 17-OHPREG and DHEA and greater access to PREG. In contrast, 3 β HSD localized to the smooth ER would presumably have greater access to the cytosolic steroid substrates and less to PREG. Such differential access to steroid substrates resulting from variations in the localization of 3 β HSD therefore has the potential to influence the steroidogenic output of the cell significantly (Chapman et al., 2005; Simard et al., 2005). This hypothesis is supported by co-localization and precipitation studies in bovine adrenocortical mitochondria that showed 3 β HSD and CYP11A1 to be in a functional steroidogenic complex in the inner mitochondrial membrane, promoting the production of PROG in these cells (Cherradi et al., 1994, 1995). Such a mechanism has also been proposed to serve as a means for the

production of high levels of PROG in the corpora lutea of pregnant mice (Chapman et al., 2005). However, the exact functional significance of differential 3 β HSD sub-cellular localization remains to be fully elucidated (Simard et al., 2005).

3.6 Transcriptional regulation of human adrenal 3 β HSD

ACTH and angiotensin II have long been known to regulate 3 β HSD expression in the human adrenal glands. Various studies have demonstrated the ability of ACTH and angiotensin II as well as the second messenger mimics of ACTH (cAMP, forskolin and cholera toxin involved in the protein kinase A signaling pathway) and angiotensin II (phorbol esters involved in the protein kinase C signaling pathway) to regulate 3 β HSD expression (Trudel et al., 1991; Naville et al., 1991; Lebrethan et al., 1994; Bird et al., 1996; Bird et al., 1998; Simard et al., 2005). However, data regarding the transcriptional regulation of 3 β HSD following trophic hormone stimulation is limited. Subsequent to the characterization of the human *HSD3B2* gene a number of studies analyzed the promoter and 5'-flanking regions of the *HSD3B2* gene searching for regulatory elements that may be involved in the transcriptional regulation of this gene. Since cAMP is a known mediator of trophic hormone stimulation, investigators expected to find cAMP response elements in the 5'-flanking region of the *HSD3B2* gene. No such elements could, however, be identified. Instead investigators identified a consensus regulatory element at -64 to -56 that binds the orphan nuclear receptor steroidogenic factor-1 (SF-1), with subsequent studies investigating the possible involvement of the SF-1 element in the regulation of human adrenal 3 β HSD expression (Leers-Sucheta et al., 1997; Simard et al., 2005; LaVoie and King, 2009). Results from these studies indicated that SF-1 was essential for cAMP and phorbol ester stimulated steroidogenesis and further suggested that trophic hormone stimulation of 3 β HSD expression involves SF-1 activation (Martin et al., 2005; LaVoie and King, 2009; Hoivik et al., 2010). However, the exact mechanism through which cAMP and phorbol esters stimulate the expression of the *HSD3B2* gene via SF-1 activation is still unclear (Simard et al., 2005).

Further analysis of the human *HSD3B2* promoter region identified a second regulatory element at -118 to -110 that interacts with the transcription factor Stat5 (Feltus et al., 1999; LaVoie and King, 2009). Stat5 is a member of the Stat (signal transducers and activators of transcription) family of proteins that function as transcription factors. Stat have a number of extracellular ligands such as cytokines, growth factors and prolactin (PRL) which activates their translocation from the cytoplasm to the nucleus through tyrosine kinase activity. Once in the nucleus these transcription factors mediate the transcription of specific target genes by binding to specific regulatory elements in the promoter region

of these genes (Darnell, 1997). Therefore, the presence of a Stat5 regulatory element in the *HSD3B2* promoter region suggests a possible regulatory role for Stat5 in the expression of human adrenal 3 β HSD (Simard et al., 2005). This has been confirmed in expression studies where PRL was shown to activate Stat5 regulation of the *HSD3B2* promoter. Disruption of the Stat5 regulatory element through site-directed mutagenesis also significantly reduced this response to PRL stimulation (Feltus et al., 1999). In addition, the presence of a Stat5 regulatory element in the promoter of the *HSD3B2* gene also opens up the possibility that this gene may be regulated by other additional ligand-stimulated pathways. Angiotensin II, for example has been shown to stimulate Stat5 in cardiac myocytes (McWhinney et al., 1998). Therefore, a similar pathway may exist in the adrenal zona glomerulosa, allowing angiotensin II to stimulate mineralocorticoid synthesis through the up regulation of 3 β HSD expression via Stat5 (Simard et al., 2005). Furthermore, epidermal growth factor (EGF) has also been shown to stimulate cortisol synthesis in H295 cells whilst also stimulating 3 β HSD promoter activity and mRNA levels. Thus, EGF may also serve as an additional ligand that utilizes the Stat5 pathway to regulate 3 β HSD expression (Feltus et al., 2003; Simard et al., 2005).

Increasing evidence is emerging that implicate steroid hormones as modulators of adrenal 3 β HSD expression (Perry and Stalvey, 1992; Stalvey and Clavey, 1992; Feltus et al., 2002). This is intriguing as the promoter and 5'-flanking region of the *HSD3B2* gene does not contain clear steroid regulatory elements. A number of possibilities have been suggested to explain this phenomenon. These include steroid hormones altering 3 β HSD expression through post-transcriptional mechanisms or by altering the transcriptional level of other transcription factors which target the 3 β HSD promoter (Simard et al., 2005). However, a growing body of evidence now suggests that the modulation of 3 β HSD expression most likely occurs through the non-classical action of the corresponding nuclear-hormone receptors. These non-classical mechanisms alter transcription through protein-protein interactions with other transcription factors without directly binding to a target sequence in the promoter region of the target gene (Funder, 2005; Good, 2007). This is supported by a number of studies that have shown steroid receptors to alter transcription in a ligand dependant manner following interaction with Stat proteins, activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B) and specificity protein-1 (Sp1) (Stocklin et al., 1996; McKay and Cidlowski, 1998; Reichardt et al., 1998; Tuckermann et al., 1999; Saville et al., 2000). Furthermore, Stat5 has also been shown to play a critical role in the stimulation of human type II 3 β HSD mRNA levels and promoter activity following glucocorticoid treatment in H295R cells (Feltus et al., 2003). The mechanism through which this stimulation occurs has yet to be fully characterized but may possibly involve protein-protein interaction between the GR and Stat5 (Simard et al., 2005).

In addition to SF-1 and Stat5 a number of other factors have been identified which influence the expression of 3 β HSD. These include GATA proteins (Tremblay and Viger, 2003; Viger et al., 2004; Martin et al., 2005), Nur77 (Bassett et al., 2004; Lu et al., 2004; Martin and Tremblay, 2005), Interleukin-4 (IL-4) and dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X-chromosome gene-1 (DAX-1) (Lalli et al., 1998; Simard et al., 2005). However, even though a number of factors have been identified which influence the expression of human adrenal 3 β HSD a significant amount of research is still needed to elucidate the precise mechanisms involved as well as the interplay between these potential regulatory pathways.

3.7 3 β HSD deficiency

3 β HSD deficiency is a form of congenital adrenal hyperplasia (CAH) that accounts for approximately 10% of all CAH cases (Bios et al., 1985; Thilen and Larsson, 1991; Simard et al., 2005). CAH describes a collective group of syndromes caused by specific steroidogenic enzyme deficiencies in the adrenal cortex. These deficiencies reduce the capacity of the adrenal cortex to produce cortisol resulting in a compensatory hypersecretion of ACTH by the anterior pituitary leading to hyperplasia of the adrenal cortex (Morel and Miller, 1991; Simard et al., 1995; Pang, 2001). The most frequent causes of CAH are mutations in the genes encoding CYP21 and CYP11B1. In contrast to CYP21 and CYP11B1 deficiency, which are exclusive to the adrenal, 3 β HSD deficiency affects steroidogenesis in both the adrenals and gonads as a result of mutations in the *HSD3B2* gene. Consequently, 3 β HSD deficiency not only impairs the synthesis of cortisol, but also of aldosterone, progesterone, androgens and estrogens resulting in diverse clinical features depending on the severity of the loss in 3 β HSD activity (Table 3.2) (Simard et al., 1995; Morel et al., 1997; Penning, 1997; Pang, 1998; Pang, 2001; Simard et al., 2005).

3 β HSD deficiency can be divided into two main forms, namely classical and non-classical 3 β HSD deficiency. The classical form of 3 β HSD deficiency is further sub-divided into salt-wasting or non-salt wasting forms, depending on the degree to which aldosterone synthesis is impaired (Simard et al., 2005 and references therein). Genetic males (XY) suffering from classical 3 β HSD deficiency normally present with either perineal hypospadias or perineoscrotal hypospadias (Table 3.2). In contrast, genetic females suffering from complete or partial inhibition of the type II 3 β HSD enzyme typically do not exhibit ambiguous external genitalia but may exhibit some clitoromegaly or slight virilization. This distinct phenotypic difference between male and female individuals suffering from classical 3 β HSD deficiency is attributed to the inhibition of 3 β HSD activity in the fetal testis. This

reduces the level of testosterone and dihydrotestosterone (DHT) below that needed for normal development of external genitalia in genetic males (Morel et al., 1997; Russell et al., 1994; Pang, 1998).

Diagnosis of the salt-wasting form of 3 β HSD deficiency is normally made during early infancy due to excessive salt-wasting, which if left untreated may be fatal (Bongiovanni, 1961, 1962; Parks et al., 1971; Heinrich et al., 1993; Simard et al., 2005). The early diagnoses of the non-salt wasting form of 3 β HSD deficiency is, however, more difficult, especially in female newborns because sexual differentiation in these individuals is usually normal. Nevertheless, early diagnosis may be made in the presence of indicating factors such as a family history of death during early infancy, failure to gain weight or perineal hypospadias in male newborns (Gendrel et al., 1979; Nahoul et al., 1989; Simard et al., 2005). Later, during early childhood or young adulthood, the non-salt wasting form of 3 β HSD deficiency may also be diagnosed in individuals who exhibit signs of premature pubarche, mild growth acceleration, clitoromegaly, hirsutism or menstrual disorders (Rosenfield, 1980; Chang et al., 1992; Pang, 1998). Biologically, classical 3 β HSD deficiency is characterized by elevated plasma levels of PREG, 17-OHPREG and DHEA. However, Δ^4 steroid plasma levels are also frequently elevated in 3 β HSD deficient patients due to the extra adrenal conversion of Δ^5 steroids by the functional 3 β HSD type I enzyme. Therefore, the best criteria for the biological diagnosis of classical 3 β HSD deficiency is considered to be an elevated ratio of Δ^5/Δ^4 steroids accompanied by a plasma level of 17-OHPREG greater than 100 nmol/l following ACTH stimulation (de Peretti and Forest, 1982; Cara et al., 1985; Pang, 2001; Simard et al., 2005).

Non-classical 3 β HSD deficiency, also referred to as late-onset or attenuated 3 β HSD deficiency, has been described in older girls and young woman with hyperandrogenism who exhibit signs of premature pubarche, menstrual irregularities and hirsutism (Simard et al., 2005; Miller and Auchus, 2010 and references therein). These individuals have a slightly elevated basal ratio of Δ^5/Δ^4 steroids that is further increased by ACTH stimulation (Rosenfield et al., 1980; Pang et al., 1983; Pang et al., 1985; Miller and Auchus, 2010). Initially, this condition was thought to be a mild variant of classical 3 β HSD deficiency resulting from mutations in the *HSD3B2* gene. However, studies investigating the molecular basis for this condition found no mutations in either genes encoding the type I or type II 3 β HSD in patients suffering from apparent non-classical 3 β HSD deficiency (Zerah et al., 1994; Chang et al., 1995; Forest et al., 1995). Therefore, the basis for hyperandrogenism and mildly elevated ratios of Δ^5/Δ^4 steroids in woman suffering from premature pubarche, menstrual irregularities and hirsutism is not yet fully known (Miller and Auchus, 2010).

Studies investigating the molecular basis for classical 3 β HSD deficiency have identified numerous mutations in the *HSD3B2* gene resulting in mutant enzymes exhibiting a complete or partial loss in 3 β HSD activity (Table 3.2). It is expected that individuals suffering from the severe salt-wasting form would express an inactive mutant type II 3 β HSD enzyme in both the gonads and adrenals whilst individuals suffering from the less severe non-salt wasting form are expected to express a mutant enzyme that retains some 3 β HSD activity (Simard et al., 2005). Indeed, studies investigating genotype-phenotype relationships in individuals suffering from classical 3 β HSD deficiency have found that in general missense mutations associated with the severe salt-wasting form exhibit no detectable enzyme activity. Furthermore, results also show that missense mutations associated with the non-salt wasting form do not completely abolish 3 β HSD activity but allows enough residual activity for sufficient aldosterone production, preventing excessive salt loss (Moisan et al., 1999; Pang, 2001; Simard et al., 2005). Therefore, even a partial loss in 3 β HSD activity as a result of mutations in the *HSD3B2* gene has a significant influence on adrenal and gonadal steroidogenesis, resulting in a wide spectrum of phenotypic manifestations. Table 3.2 highlights several mutations in the *HSD3B2* gene to illustrate the variety of functional and phenotypic consequences that such mutations have in individuals suffering from 3 β HSD deficiency.

Table 3.2 Mutations in the HSD3B2 gene resulting in the salt-wasting or non-salt wasting forms of classical 3 β HSD deficiency depending on the degree of 3 β HSD inhibition. Adapted from Simard et al (2005).

Sex	Phenotype	Mutation	Apparent activity	Reference
<i>Salt-wasting</i>				
Male	Ambiguous genitalia Palpable testis in bifid scrotum	A10E	None detectable	Alos et al., 2002
Male	Perineoscrotal hypospadias Bifid scrotum	G15D	None detectable	Gendrel et al., 1979 Rheaume et al., 1995
Male	Hyperpigmentation Severe hypospadias Bifid scrotum	L205P	None detectable	Moisan et al., 1999 Katsumata et al., 1995
Female	Mild clitoromegaly	P222Q	None detectable	Moisan et al., 1999
Female	Normal genitalia with severe pigmentation	T295R	None detectable	Moisan et al., 1999 Tajima et al., 1995
<i>Non-Salt wasting</i>				
Male	Hypospadias Hyperpigmented bifid scrotum	L6F	54.75 \pm 8%	Zang et al., 2000
Male	Perineoscrotal hypospadias	A10V	29.1 \pm 0.6%	Moisan et al., 1999
Male	Scrotal hypospadias Bifid scrotum	A245P	35.5 \pm 0.2 %	Simard et al., 1993b Heinrich et al., 1993
Female	Normal genitalia Premature acne	E142K	27%	Pang et al., 2002
Male	Perineal hypospadias	G294V	20.5 \pm 4.6 %	Moisan et al., 1999 Gendrel et al., 1979

3.8 Summary

From the preceding discussion it is clear that 3 β HSD plays a key role in maintaining normal physiological processes through the synthesis of steroid hormones in a variety of different tissues, including the adrenals, testis and ovaries. Consequently, variations in 3 β HSD activity have the potential to significantly influence various physiological and developmental processes. Indeed, studies investigating classical 3 β HSD deficiency in humans have shown this to be true as even partial inhibition of 3 β HSD activity results in a variety of clinical disorders. These disorders result from the insufficient synthesis of steroid hormones such as cortisol, aldosterone and testosterone with the severity of the symptoms depending on the degree to which 3 β HSD activity is impaired (Moisan et al., 1999; Pang, 2001; Simard et al., 2005). Alterations in 3 β HSD activity therefore have the potential to directly impact various physiological and developmental processes by altering the synthesis of the steroid hormones which mediate these processes. The manner in which variations in 3 β HSD activity

alter the biosynthesis of these hormones is, however, unclear. It is known that 3 β HSD and CYP17 compete for the Δ^5 steroid intermediates, PREG and 17-OHPREG during adrenal steroidogenesis. Nevertheless, limited experimental data are available describing the influence that varying levels of 3 β HSD activity has on this competition and how fluctuations in the outcome of this competition may ultimately impact the biosynthesis of active steroid hormones downstream. To effectively investigate the influence of 3 β HSD activity on adrenal steroidogenesis requires the use of a relevant experimental model. Such a model should exhibit a known steroidogenic deficiency that may result from altered 3 β HSD activity levels. One such model is the South African Angora goat, known to produce insufficient levels of cortisol (Van Rensburg, 1971; Engelbrecht et al., 2000). The following chapter will describe the South African Angora goat as an experimental model to investigate the influence of variations in 3 β HSD activity on adrenal steroidogenesis and how such variations may ultimately influence cortisol production.

CHAPTER 4

HYPOCORTISOLISM IN THE SOUTH AFRICAN ANGORA GOAT: THE ROLE OF 3 β HSD

4.1 Introduction

The discussions presented in the preceding chapters clearly indicate that the catalytic activity of 3 β HSD has the potential to significantly influence the steroidogenic output of the adrenal. Nevertheless, the manner in which alterations in 3 β HSD activity alters the flux of steroid intermediates, and ultimately the synthesis of active steroid hormones, remains unclear. The aim of the study presented in this chapter, was therefore to investigate the competitive interaction of 3 β HSD and CYP17 in the metabolism of PREG, and the impact of this competition on the flux of steroid intermediates during adrenal steroidogenesis. In this study 3 β HSD from two different small stock species, the South African Angora goat and Merino sheep, were compared. These animals were chosen as the enzyme differs by five amino acid residues between the species. The influence of these differences on the catalytic activity of 3 β HSD and on the competition between 3 β HSD and CYP17 for the same substrates were examined.

The South African Angora goat was chosen as a relevant experimental model since the specie is characterized by a known steroidogenic deficiency. These animals are extremely susceptible to cold stress due to a diminished capacity to produce cortisol. Previous studies have identified CYP17 as a causative factor of hypocortisolism in these animals. However, the contribution of 3 β HSD in causing this condition was uncertain (Van Rensburg, 1971; Wentzel et al., 1979; Engelbrecht et al., 2000; Storbeck et al., 2007). The South African Angora goat therefore provided an excellent model in which to study the influence of 3 β HSD activity on adrenal steroidogenesis, and more specifically cortisol biosynthesis.

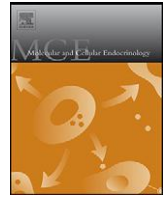
This chapter describes a study in which the cDNA sequence and kinetic parameters of Angora 3 β HSD were characterized and compared to ovine 3 β HSD, which is generally considered more hardy against cold stress. Following characterization, both Angora and ovine 3 β HSD were individually coexpressed with Angora CYP17 in COS-1 cells and the activity assayed with PREG as substrate. The resulting steroid metabolites were characterized and compared to ascertain how differences in 3 β HSD activity between these species influence the flux of steroid intermediates through the adrenal

steroidogenic pathway and ultimately cortisol production. These results were published in *Molecular and Cellular Endocrinology* and are presented in the article included in this chapter (Goosen et al., 2010). [Reprinted from *Molecular and Cellular Endocrinology*, Pierre Goosen, Amanda C. Swart, Karl-Heinz Storbeck, Pieter Swart, Hypocortisolism in the South African Angora goat: The role of 3 β HSD, 182-187, 2010, with permission from Elsevier, license no. 3000030313862]



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ABSTRACT

South African Angora goats are susceptible to cold stress, due to their inability to produce sufficient levels of cortisol. During adrenal steroidogenesis the production of cortisol relies on the activity of two key enzymes, namely cytochrome P450 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase. Cytochrome P450 17 α -hydroxylase has previously been identified as a factor contributing to hypocortisolism in the South African Angora goat. In this comparative study, the catalytic activity of Angora and ovine 3 β -hydroxysteroid dehydrogenase, which differ by five amino acid residues, was characterized. The conversion of 17-hydroxypregnenolone and dehydroepiandrosterone to their corresponding products, 17-hydroxyprogesterone and androstenedione, by the two enzymes differed significantly. The enzymes were subsequently co-expressed with Angora P450 17 α -hydroxylase. Major differences were observed in pregnenolone metabolism with a significant reduction in the formation of the cortisol precursor, 17-hydroxyprogesterone, by cells expressing Angora 3 β -hydroxysteroid dehydrogenase, implicating 3 β -hydroxysteroid dehydrogenase as an additional factor contributing to hypocortisolism in the South African Angora goat.

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1. Introduction

In South Africa, Angora goats are an important fiber-producing small stock breed, which produce some of the highest quality mohair in the world. As such, the South Africa mohair industry meets close to 60% of the world's mohair demand. Unfortunately, high quality fleece has been found to be negatively related to fitness traits (Snyman and Olivier, 1996), making the South African Angora goat extremely susceptible to cold stress, which results in frequent and severe losses of young and newly shorn goats during cold spells.

The Angora goat is unable to metabolize glucose during prolonged periods of cold exposure, thereby reducing its ability to produce sufficient metabolic heat (Wentzel et al., 1974, 1979). Engelbrecht et al. (2000) attributed this to a condition of hypocortisolism since *in vivo* stimulation of the Angora hypothalamic–pituitary–adrenal (HPA) axis with insulin and ACTH resulted in lower cortisol production by Angora goats relative to Boer goats and Merino sheep. These findings supported data previously obtained by Van Rensburg (1971), who showed that selective breeding for high quality fleece resulted in reduced adrenal function in the Angora goat characterized by a decrease in cortisol production.

During adrenal steroidogenesis the production of cortisol is influenced by the activity of two key enzymes, 3 β -hydroxysteroid dehydrogenase/ Δ^5 – Δ^4 isomerase (3 β HSD) and cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17), which compete for the same Δ^5 steroid substrates (Simard et al., 2005; Payne and Hales, 2004). 3 β HSD is a bifunctional enzyme, involved in the production of all classes of steroid hormones by catalyzing the conversion of Δ^5 steroid intermediates to their corresponding Δ^4 isoforms through sequential dehydrogenase and isomerase reactions (Van et al., 1991; Thomas et al., 1995). In contrast, CYP17 is a hemoprotein responsible for the 17 α -hydroxylation of pregnenolone (PREG) and progesterone (PROG) yielding 17-hydroxypregnenolone (17-OHPREG) and 17-hydroxyprogesterone (17-OHPROG), respectively. In addition the 17,20 lyase reaction of CYP17 catalyzes the cleavage of the C17,20 bond of 17-OHPREG and 17-OHPROG yielding dehydroepiandrosterone (DHEA) and androstenedione (A4), respectively (Nakajin and Hall, 1981; Nakajin et al., 1981; Zuber et al., 1986). Due to the competition between 3 β HSD and CYP17 for Δ^5 steroid substrates, the activity of these enzymes play a critical role in the steroidogenic output of the adrenal cortex.

Engelbrecht and Swart (2000) demonstrated that Angora CYP17 has a greater activity towards PREG when compared to the Boer goat and Merino sheep enzymes, the latter two breeds generally being considered more hardy. In contrast, the conversion of PREG by 3 β HSD was found to be similar for all three species. From these results Engelbrecht and Swart (2000) concluded that increased CYP17 activity was a factor in causing hypocortisolism by altering the flux of steroid intermediates through the Δ^5 steroid pathway.

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Storbeck et al. (2007) subsequently identified two isoforms of Angora CYP17, denoted as ACS+ and ACS–. These isoforms were shown to differ in their 17,20 lyase activity towards 17-OHPREG with ACS– exhibiting a significantly greater 17,20 lyase activity towards 17-OHPREG than ACS+. Both isoforms were also shown to be unable to metabolize 17-OHPREG to A4. Furthermore, the difference in 17,20 lyase activity was demonstrated to increase the flux of intermediates through the Δ^5 pathway in ACS– animals (Storbeck et al., 2007). These data supported the previous findings by Engelbrecht and Swart (2000) and implicated the activity of CYP17 as the primary cause of hypocortisolism in the South African Angora goat. However, the study conducted by Engelbrecht and Swart (2000) did not investigate 3 β HSD activity towards 17-OHPREG or DHEA since the adrenal microsomal preparations in which the assays were conducted not only contained 3 β HSD but also various other steroidogenic enzymes and cofactors, complicating the interpretation of data. Thus, the possible contribution of 3 β HSD towards hypocortisolism in the Angora goat remained uncertain.

In this study, Angora and ovine 3 β HSD cDNAs were cloned and sequenced. The enzymes were expressed in nonsteroidogenic COS-1 cells and the catalytic activities towards PREG, 17-OHPREG and DHEA assayed in the absence of other steroidogenic enzymes. In addition, both Angora and ovine 3 β HSD were co-expressed with Angora CYP17 to ascertain whether 3 β HSD is a potential contributing factor in causing hypocortisolism in the South African Angora goat.

2. Experimental procedures

2.1. Materials

An mRNA capture kit and Titan™ One Tube RT-PCR system was purchased from Roche Applied Science (Mannheim, Germany). Plasmid vectors and restriction enzymes were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) and Roche Applied Science (Mannheim, Germany), respectively. Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Nucleobond® AX plasmid purification kits were supplied by Macherey–Nagel (Duren, Germany) and Wizard® Plus SV Minipreps DNA Purification kits were purchased from Promega Biotech (Madison, WI, USA). COS-1 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum and bacterial culture medium were purchased from Highveld Biological (Lyndhurst, SA) and Difco Laboratories (Detroit, MI, USA), respectively. Penicillin–streptomycin, trypsin–EDTA and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). AMV Reverse Transcriptase and an Oligo(dT)15 primer were purchased from Promega (Madison, WI, USA). LightCycler® FastStart DNA Master SYBR Green 1 was supplied by Roche Applied Science (Mannheim, Germany). [1,2,6,7-³H]-DHEA and [7-³H]-PREG were purchased from PerkinElmer Life Sciences (Boston, MA, USA). PREG, 17-OHPREG, DHEA, Dulbecco's modified Eagle's medium (DMEM) and TRI Reagent® solution were supplied by Sigma Chemical Co. (St. Louis, MO, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest quality and obtained from reputable scientific supply houses.

2.2. cDNA preparation

Angora and ovine adrenal glands were obtained at a local abattoir and flash frozen in liquid N₂. Ovine adrenal cortex homogenate was prepared using multiple adrenal glands collected from various ovine sub-species, which varied in age and sex. Angora adrenal cortex homogenate was prepared using adrenal glands from animals which varied in age and sex. mRNA was isolated from Angora and ovine adrenal cortex homogenate using a mRNA capture kit (Roche), according to manufacturer's instructions. cDNA was synthesized using the Titan™ One Tube RT-PCR system (Roche) and the PCR products cloned into the pcDNA/V5/GW/D-TOPO® mammalian expression vector system (Invitrogen), according to manufacturer's instructions. The primers used to amplify Angora goat and ovine 3 β HSD were as follows: sense primer, 5'-CACCTGGCTTCCACAATC-3'; antisense primer, 5'-CGGAAGCGAGATAGTAAGG-3'. Plasmid constructs containing 3 β HSD cDNA were screened by restriction digestion using the restriction enzyme AclI (Roche). Positive clones were subsequently subjected to direct DNA sequence analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Johannesburg, RSA).

2.3. Angora and ovine 3 β HSD enzyme activity assay in transiently transfected COS-1 cells

COS-1 cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin and 0.12% NaHCO₃. Confluent cells were plated into 12-well plates, 1.0×10^5 cells/ml, 24 h prior to transfection. COS-1 cells were transiently transfected with either the Angora or ovine 3 β HSD plasmid constructs (0.5 μ g) using TransIT®-LT1 transfection reagent (Mirus), according to manufacturer's instructions. Control transfection reactions were performed using the mammalian expression vector pCIneo (Promega, Madison, WI, USA) containing no insert. In addition, Angora and ovine 3 β HSD (0.5 μ g) were transiently cotransfected with Angora CYP17 (0.5 μ g) as described above.

Enzyme activity was assayed after 72 h by adding 1 μ M PREG and [³H]PREG; 17-OHPREG; DHEA and [³H]DHEA as substrates, respectively. At specific time intervals aliquots were removed from the assay mixtures—PREG (50 μ l), DHEA (50 μ l) and 17-OHPREG (500 μ l). The steroid metabolites were subsequently extracted by liquid–liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 15 min and centrifuged at 500 \times g for 5 min. The water phase was aspirated off, and the dichloromethane phase transferred to a clean test tube and dried under N₂. The dried steroid residue was redissolved in 120 μ l methanol prior to HPLC or UPLC–MS analysis. After completion of each experiment, the cells were washed and collected in phosphate buffer (0.1 M, pH 7.4). The cells were homogenized with a small glass homogenizer, and the total protein content of the homogenate was determined by the Pierce BCA method according to manufacturer's instructions.

2.4. Separation and quantification of steroids

High performance liquid chromatography was performed on a SpectraSYSTEM P4000 high performance liquid chromatograph (Thermo Separation™ products, San Jose, CA, USA) coupled to a SpectraSYSTEM AS3000 automatic injector (Thermo Separation™ products, San Jose, CA, USA) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA). The substrates, PREG and DHEA and their respective steroid metabolites were separated using a Phenomenex® C₁₂ column at a flow rate of 1 ml min^{−1}. The ratio of scintillant to column element was 3:1. For the separation of PREG from PROG the mobile phase consisted of solvent A (methanol/water: 75%/25%) and solvent B (100% methanol). The column was eluted for 2 min with solvent A, followed by a linear gradient from 100% A to 100% B in 9 min and an isocratic elution with solvent B for 2 min. A linear gradient returned the column to 100% A in 3 min. The total run time per sample was 16 min and the injection volume of each sample was 90 μ l. DHEA and A4 were eluted with solvent A (methanol/water: 65%/35%) for 15 min, followed by a linear gradient from 100% A to 100% B (100% methanol) in 2 min and an isocratic elution with solvent B for 2 min. A linear gradient returned the column to 100% A in 3 min. The total run time per sample was 21 min and the injection volume of each sample was 90 μ l.

17-OHPREG and 17-OHPREG were analyzed by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Waters UPLC BEH C₁₈ column (2.1 mm \times 50 mm, 1.7 μ m) at 50 °C and a flow rate of 0.4 ml min^{−1} as previously described (Storbeck et al., 2008). PREG metabolites were separated using a Waters UPLC BEH C₁₈ column (2.1 mm \times 100 mm, 1.7 μ m) at 50 °C and a flow rate of 0.3 ml min^{−1} as previously described (Storbeck et al., 2008). An API Quattro Micro tandem mass spectrometer (Waters, Milford, MA, USA) was used for quantitative mass spectrometric detection as previously described (Storbeck et al., 2008).

2.5. RNA isolation and cDNA synthesis from COS-1 cells

Total RNA was isolated from COS-1 cells, expressing either Angora or ovine 3 β HSD, in duplicate using TRI Reagent® solution (Sigma) according to the manufacturer's instructions. mRNA was subsequently reverse transcribed using AMV Reverse Transcriptase (Promega) for each replicate according to the manufacturer's instructions. The synthesised cDNA was subsequently used at a 1000 \times dilution in the real-time amplification reactions.

2.6. Real-time PCR

Primers for 3 β HSD and a reference gene, β -Actin, designed to have similar melting temperatures and product sizes, were: 3 β HSD, sense primer 5'-CGGCA TCCTGACCAATTAC-3', antisense primer 5'-GGCCACATTGCCAACATAGA-3'; and β -Actin, sense primer 5'-CCCACACTGTGCCCATCTA-3', antisense primer 5'-CGTGGTGGTGAAGCTGTA-3' (Metabion international AG, Martinsried, Germany). The 3 β HSD primers amplified a 64 bp region identical in both Angora and ovine 3 β HSD cDNA sequences. Real-time PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20 μ l) contained 3 mM MgCl₂, 2 μ l LightCycler® FastStart DNA Master SYBR Green 1 (Roche), 0.5 μ M of either 3 β HSD or β -Actin primer and 2 μ l cDNA sample. Following an initial denaturation step at 95 °C for 10 min, the 35 cycle amplification profile consisted of heating to 95 °C with an 10 s hold, cooling to 52 °C with an 10 s hold and heating to 72 °C with a 6 s hold. The transition rate between all steps was 20 °C s^{−1}. Data were acquired in single mode during the 52 °C phase using LightCycler® software (version 3.5).

Table 1Differences in primary structure between Angora and ovine 3 β HSD.

Position	Angora 3 β HSD residue	Ovine 3 β HSD residue
17	Gln	Arg
73	Cys	Phe
100	Ile	Val
153	Ser	Cys
336	Arg	Gln

Following amplification a melting curve analysis was performed as follows: denaturation at 95 °C with a 0 s hold, annealing at 65 °C with a 15 s hold followed by heating to 95 °C at a rate of 0.1 °C s⁻¹ with continuous data acquisition. Both the target 3 β HSD genes and the reference gene were amplified independently and in duplicate during the same experimental run for each cDNA sample. A negative control containing no template cDNA was also included. Melting curve analysis showed all reactions were free of primer dimers and other non-specific products. Serial dilutions were performed in triplicate and used to determine the PCR efficiencies of both the target and reference genes. The PCR efficiencies were calculated from the slopes of standard curves generated by the LightCycler® software and were always >92%. C_t values were generated for both the target and reference genes in each sample using the second-derivative maximum mode of analysis. ΔC_t values were calculated on the basis of the mean C_t values for both target genes and the reference gene ($\Delta C_{t, \text{Angora/ovine}} = \text{Mean } C_{t, 3\beta\text{HSD}} - \text{Mean } C_{t, \beta\text{-Actin}}$). The $\Delta\Delta C_t$ value was calculated on the basis of the mean ΔC_t values for both Angora and ovine 3 β HSD ($\Delta\Delta C_t = \text{Mean } \Delta C_{t, \text{Angora}} - \text{Mean } \Delta C_{t, \text{ovine}}$). The mean fold change in the amount of starting mRNA template in cells expressing either Angora or ovine 3 β HSD was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Angora and ovine 3 β HSD cDNA sequence analyses

Direct DNA sequence analysis of the RT-PCR product revealed 97% sequence identity between the Angora (GenBank accession no. EF524065) and ovine 3 β HSD (GenBank accession no. FJ007375) cDNA sequences. As previously mentioned, the ovine 3 β HSD cDNA sequence was prepared using adrenal tissue collected from various animals, suggesting that the sequence represents wild type ovine 3 β HSD. Translation of these sequences identified five differences in primary structure of which four are non-conservative (Table 1). The amino acid residues at positions 17 and 73 are located in a nucleotide-binding site sequence, a β -strand, α -helix, β -strand common to all members of the short-chain alcohol dehydrogenase superfamily. The residue at position 153 is located adjacent to the putative catalytic motif Y¹⁵⁴-X-X-X-K¹⁵⁸ (Simard et al., 2005).

3.2. Angora and ovine 3 β HSD activity in COS-1 cells

Both Angora and ovine 3 β HSD were expressed in nonsteroidogenic COS-1 cells and assayed for activity with PREG, 17-OHPREG

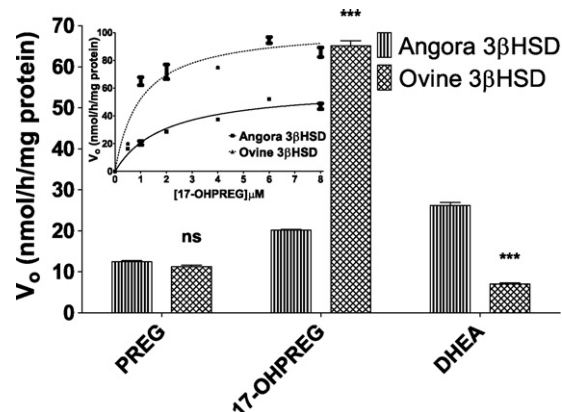


Fig. 1. Comparison of the initial reaction rates of Angora and ovine 3 β HSD for the conversion of 1 μ M PREG, 17-OHPREG and DHEA. V_0 of Angora 3 β HSD is compared independently to that of ovine 3 β HSD for each substrate using an unpaired t -test ($p > 0.05$, ns; $p < 0.01$, **; $p < 0.001$, ***; $n = 3$) Insert: Michaelis–Menten plots of 17-OHPREG metabolism by Angora and ovine 3 β HSD expressed in COS-1 cells.

and DHEA as substrates. Similar initial reaction rates were obtained for both enzymes during the conversion of PREG as previously demonstrated by Engelbrecht and Swart (2000). However, significant differences in the initial rate of conversion were observed for 17-OHPREG and DHEA metabolism. The initial reaction rate of ovine 3 β HSD for the conversion of 17-OHPREG was significantly greater ($p < 0.001$) than that of the Angora enzyme. In contrast, the activity of Angora 3 β HSD towards DHEA was significantly greater ($p < 0.001$) than that of ovine (Table 2, Fig. 1).

Apparent K_m and V_{max} values of Angora and ovine 3 β HSD towards PREG, 17-OHPREG and DHEA were subsequently determined. Michaelis–Menten plots (not shown) were obtained for each K_m and V_{max} determination, showing saturation was reached for each enzyme towards the three respective substrates. These data (Table 3) correlate with the initial reaction rates (Table 2) showing that Angora 3 β HSD had a greater affinity for DHEA as was reflected in the K_m value of 1.3 μ M. In contrast, ovine 3 β HSD had a greater affinity for 17-OHPREG with a K_m value of 0.9 μ M.

3.3. PREG metabolism by Angora and ovine 3 β HSD in the presence of CYP17

In the adrenal gland CYP17 and 3 β HSD compete for the same Δ^5 steroid substrates. CYP17 and 3 β HSD were therefore cotransfected into COS-1 cells to determine whether differences observed in the catalytic activity between the Angora and ovine 3 β HSD

Table 2

Initial reaction rates (V_0) of Angora and ovine 3 β HSD for the conversion of 1 μ M PREG, 17-OHPREG and DHEA. The rates were determined by linear regression of at least four time points for each substrate. The R -squared value for all initial rate regression was always higher than 0.97. V_0 is expressed as the mean \pm SEM of three independent experiments.

Species	PREG V_0 (nmol/h/mg protein)	17-OHPREG V_0 (nmol/h/mg protein)	DHEA V_0 (nmol/h/mg protein)
Angora 3 β HSD	12.5 \pm 0.4	20.2 \pm 0.2	26.2 \pm 1.2
Ovine 3 β HSD	10.9 \pm 0.1	65.1 \pm 2.1	6.9 \pm 0.2

Table 3

Angora and ovine 3 β HSD apparent K_m and the V_{max} values for PREG, 17-OHPREG and DHEA. The apparent K_m (μ M) and the V_{max} values (nmol/h/mg protein) are expressed as the mean \pm SEM of three independent experiments. The apparent K_m values of Angora 3 β HSD are compared independently to that of ovine 3 β HSD for each substrate using an unpaired t -test.

Species	PREG		17-OHPREG		DHEA	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Angora	2.6 \pm 0.6	52.2 \pm 4.5	1.9 \pm 0.5	60.8 \pm 6.2	1.3 \pm 0.1	59.7 \pm 1.8
Ovine	4.7** \pm 0.2	61.6 \pm 1.7	0.9* \pm 0.4	104.6 \pm 12.3	1.8* \pm 0.2	22.5 \pm 0.9

* $p < 0.05$.** $p < 0.01$.

enzymes (Fig. 1) could possibly influence 17-OHPROG and A4 production. Both Angora and ovine 3 β HSD were cotransfected with Angora CYP17 ACS-. Cells expressing Angora 3 β HSD showed distinct differences in the production of PROG, 17-OHPROG and A4 when compared to cells expressing ovine 3 β HSD (Figs. 2 and 3). After 6 h cells cotransfected with Angora 3 β HSD and CYP17 ACS- metabolized PREG substrate to PROG ($\approx 30\%$), 17-OHPROG ($\approx 54\%$) and A4 ($\approx 2\%$). PREG metabolism by cells cotransfected with ovine 3 β HSD and Angora CYP17 ACS- was distinctly different, with PREG metabolites, PROG ($\approx 22\%$) being lower and 17-OHPROG ($\approx 68\%$) and A4 ($\approx 5\%$) being higher. 17-OHPREG and DHEA intermediates were not detected in either of the metabolic assays. Furthermore, no significant difference was observed in the percentage PREG remaining after 6 h for both Angora and ovine 3 β HSD, indicating that the differences observed in steroid output are not due to differences in the rate of PREG utilization.

3.4. Real-time PCR

Real-time PCR was employed to investigate transcriptional levels of Angora and ovine 3 β HSD in COS-1 cells. cDNA was synthesized using mRNA isolated from cells expressing either Angora or ovine 3 β HSD. Real-time PCR was subsequently performed, which revealed Angora and ovine 3 β HSD to have very similar mean C_t val-

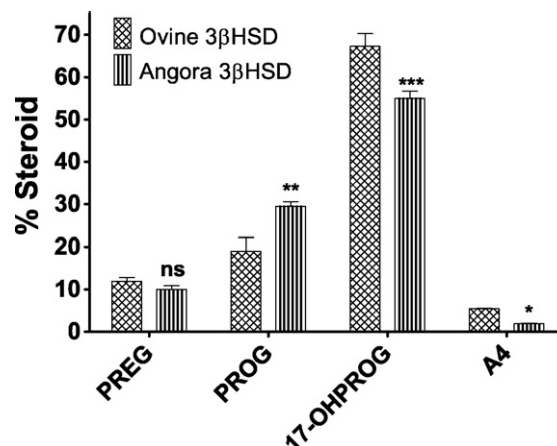


Fig. 2. Steroid profile of PREG (1 μ M) metabolism after 6 h by Angora and ovine 3 β HSD co-expressed with Angora CYP17 ACS-. The percentage of each steroid metabolite produced relative to total steroid output following the metabolism of PREG is depicted. Results are representative of three individual experiments, each performed in triplicate. Individual steroids were compared for each construct using an unpaired *t*-test ($p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $n = 3$).

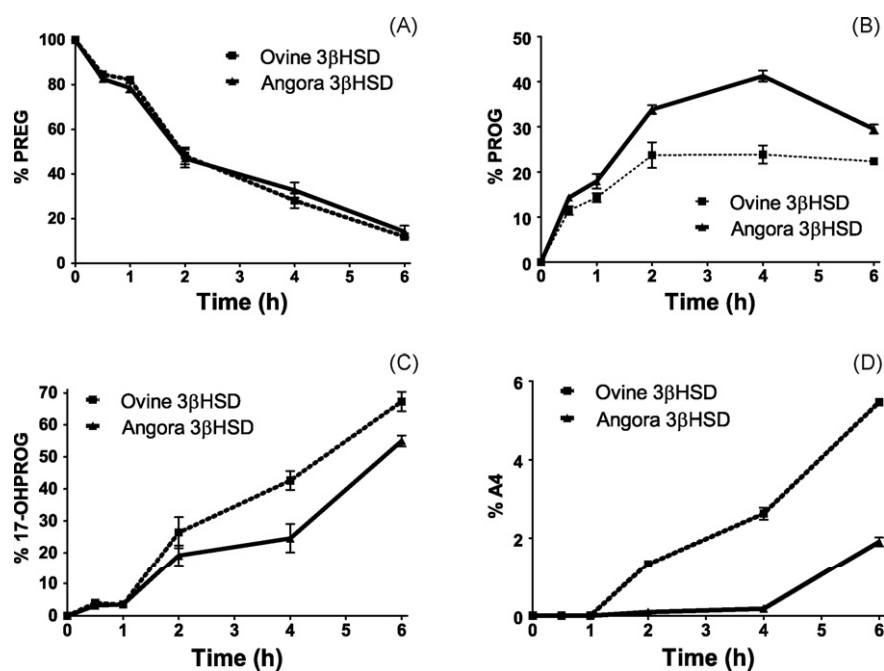


Fig. 3. Time course of PREG (1 μ M) metabolism by Angora and ovine 3 β HSD co-expressed with Angora CYP17 ACS-. The percentage of each steroid metabolite PROG (B), 17-OHPROG (C) and A4 (D) produced relative to total steroid output following the metabolism of PREG (A) is depicted. Results are representative of three individual experiments, each performed in triplicate.

Table 4

Calculation of mean fold change in amount of 3 β HSD mRNA transcript relative to β -Actin. Real-time PCR amplification performed individually for each of the target and reference genes. Mean C_t values for each target and reference gene expressed as mean \pm SEM ($n = 4$). Variance is carried forward through the calculation by using standard propagation of error methods (Livak and Schmittgen, 2001).

Template	Mean C_t	ΔC_t^a	$\Delta \Delta C_t^b$	$2^{-\Delta \Delta C_t}$
Angora 3 β HSD	19.61 \pm 0.73	–	–	–
β -Actin ^c	24.62 \pm 0.45	–5.01 \pm 0.85	–	–
Ovine 3 β HSD	19.68 \pm 0.28	–	0.27 \pm 0.92	–
β -Actin ^d	24.96 \pm 0.26	–5.28 \pm 0.37	–	0.82 \pm 0.31

^a ΔC_t , Angora/ovine = Mean C_t , 3 β HSD – Mean C_t , β -Actin.

^b $\Delta \Delta C_t$ = Mean ΔC_t , Angora – Mean ΔC_t , ovine.

^c β -Actin amplified from COS-1 cells expressing Angora 3 β HSD.

^d β -Actin amplified from COS-1 cells expressing ovine 3 β HSD.

ues. Furthermore, similar mean C_t values were observed for β -Actin (reference gene) amplified from cells expressing either Angora or ovine 3 β HSD (Table 4). These values were subsequently used to calculate the mean fold change in amount of starting mRNA transcript between Angora and ovine 3 β HSD, using the $2^{-\Delta\Delta C_t}$ method. The mean fold change was found to be $0.82 (\pm 0.31)$ (Table 4) indicating that Angora and ovine 3 β HSD mRNA transcript levels in COS-1 cells are similar.

4. Discussion

Engelbrecht et al. (2000) had previously attributed the susceptibility of the South African Angora goat to cold stress to hypocortisolism. During these studies, Angora goat adrenal steroidogenesis was shown to differ significantly from that of the Boer goat and Merino sheep. In adrenal microsomal preparations only 35% glucocorticoid precursors were produced by the Angora goat compared to the 78% and 82% produced by the Boer goat and Merino sheep, respectively. The Angora was also shown to be the only species producing A4 (Engelbrecht et al., 2000). Engelbrecht and Swart (2000) hypothesized that it was due to the different catalytic activities of the steroidogenic enzymes which resulted in a higher flux of steroid intermediates through the Δ^5 pathway leading to a subsequent decrease in cortisol production in the Angora goat.

Engelbrecht and Swart (2000) subsequently investigated Angora goat steroidogenic enzyme activity and identified CYP17 and 3 β HSD as having a potential role in hypocortisolism. A comparative study between the Angora goat, the Boer goat and the Merino sheep was again conducted, which showed Angora CYP17 to have a higher activity towards PREG whilst the activity of 3 β HSD towards PREG was similar between all three species. It was subsequently concluded that increased CYP17 activity contributed towards hypocortisolism by altering the flux of intermediates through the steroidogenic pathways, leading to a higher flux of steroid intermediates through the Δ^5 pathway (Engelbrecht et al., 2000; Engelbrecht and Swart, 2000). Storbeck et al. (2007) subsequently attributed the increased CYP17 activity to an increased 17,20 lyase activity towards 17-OHPREG by the CYP17 ACS– enzyme, further implicating CYP17 as the primary cause of hypocortisolism in the South African Angora goat. However, the activity of 3 β HSD towards the 17-OHPREG and DHEA intermediates had not been determined and thus the possible involvement of 3 β HSD in hypocortisolism not fully examined.

In an effort to establish whether 3 β HSD contributes to the observed hypocortisolism, Angora 3 β HSD was compared to ovine 3 β HSD, which is generally considered a hardy species and not susceptible to cold stress. Angora and ovine 3 β HSD cDNA, were characterized and expressed in COS-1 cells to determine and compare the activity of each enzyme towards PREG, 17-OHPREG and DHEA.

Similar initial reaction rates were observed for both Angora and ovine 3 β HSD during the conversion of PREG (Table 2, Fig. 1), confirming previous results. However, the initial reaction rate of ovine 3 β HSD for the conversion of 17-OHPREG was significantly greater ($p < 0.001$) than that of the Angora, whilst the initial reaction rate of Angora 3 β HSD towards DHEA was significantly greater than that of ovine ($p < 0.001$). The apparent K_m and V_{max} values (Table 3) of each enzyme for the three substrates reflected the initial reaction rates (Table 2). The differences in primary sequence could influence the structural fold of 3 β HSD thus, influencing the affinities of the two enzymes for PREG metabolites—apparent K_m values of 1.9 and $0.9 \mu\text{M}$ for 17-OHPREG and 1.3 and $1.8 \mu\text{M}$ for DHEA by Angora and ovine 3 β HSD, respectively. Although it is possible that the different amino acid residues, N17R, C73P and S153C, could affect the catalytic activity of the two enzymes, the bifunctional nature

of 3 β HSD (Van et al., 1991) complicates the correlation of these specific residues to either the dehydrogenase and/or isomerase activities.

According to the model proposed by Thomas et al. (1995), 3 β HSD catalyzes the conversion of Δ^5 steroid intermediates to their corresponding Δ^4 isoforms through a two steps sequential reaction. Following the first dehydrogenation reaction, both the Δ^{5-3} keto intermediate and NADH remain bound to the enzyme. The bound NADH then induces conformational changes in the enzyme resulting in the isomerization of the bound Δ^{5-3} keto intermediate to form the corresponding Δ^4 isoform. The differences in primary structure between Angora and ovine 3 β HSD – residues at positions 17 and 73, lying in the predicted redox partner binding site, and residue 153 lying next to the putative catalytic motif, Y¹⁵⁴-X-X-X-K¹⁵⁸ (Simard et al., 2005) – could influence enzyme activity in a number of ways. These include: primary substrate binding (Δ^5 substrate), dehydrogenase activity, secondary substrate binding (Δ^{5-3} keto intermediate), isomerase activity and cofactor binding (Thomas et al., 1989, 1995, 2003, 2005). Thus, attributing these kinetic data to specific residues requires the employment of techniques such as site-directed mutagenesis and homology modeling.

The increased activity of Angora 3 β HSD towards DHEA as well as its increased availability during adrenal steroidogenesis, as a result of the increased 17,20 lyase activity of CYP17 ACS– towards 17-OHPREG (Storbeck et al., 2007), appears to be a feasible explanation for the Angora goat being the only species to produce A4 (Engelbrecht et al., 2000). In addition, the combination of the increased 17,20 lyase activity of CYP17 ACS– and decreased 3 β HSD activity towards 17-OHPREG would also contribute considerably to the lower production of glucocorticoid precursors by the Angora goat compared to that of the Boer goat and Merino sheep, implicating 3 β HSD as a contributing factor in the observed hypocortisolism.

As previously shown, the majority of steroid intermediates are committed to the Δ^5 pathway in the South African Angora goat (Engelbrecht et al., 2000; Engelbrecht and Swart, 2000; Storbeck et al., 2007). 3 β HSD activity, thus becomes essential for the production of cortisol by converting 17-OHPREG to its corresponding Δ^4 isomer, 17-OHPROG. However, the significantly lower 3 β HSD activity towards 17-OHPREG shown in this study, could impair the adequate formation of 17-OHPROG, co-implicating 3 β HSD in hypocortisolism.

It is possible that if Angora 3 β HSD catalyses the metabolism of 17-OHPREG at a lower rate than ovine 3 β HSD *in vivo*, the enzyme would be a contributing factor in causing hypocortisolism. PREG metabolism was therefore assayed in non-steroidal mammalian cells. In COS-1 cells cotransfected with Angora 3 β HSD and CYP17 ACS– significantly less 17-OHPROG ($p < 0.001$) and more PROG ($p < 0.01$) was produced than in cells cotransfected with ovine 3 β HSD and CYP17 ACS– (Figs. 2 and 3). These results implicate 3 β HSD as a possible contributing factor in causing hypocortisolism in the South African Angora goat.

Since inherent differences in the expression levels of Angora and ovine 3 β HSD may influence the outcome of PREG metabolism, the levels of expression were subsequently investigated in COS-1 cells. Evaluating enzyme expression levels using techniques such as Western blot analyses are challenging as recombinant enzymes are expressed at relatively low levels in COS-1 cells. The expression of Angora and ovine 3 β HSD was therefore investigated at the transcriptional level using real-time PCR. The results (Table 4) indicated that both enzymes are transcribed at a similar level. Whilst similar transcription levels do not necessarily equate to similar protein concentrations, these results, together with the fact that PREG is utilized at a similar rate by COS-1 cells cotransfected with either Angora or ovine 3 β HSD, provides strong evidence that these enzymes are expressed at similar levels. Thus, it can be concluded

that the differences observed in PREG metabolism (Figs. 2 and 3) is not due to inherent differences in expression levels between Angora and ovine 3 β HSD but rather to the differences observed in catalytic activity.

These findings clearly identify 3 β HSD as a contributing factor in causing hypocortisolism in the South African Angora goat. The increased 17,20 lyase activity of CYP17 ACS— towards 17-OHPREG results in a shift in the flux of steroid intermediates, committing the majority of intermediates to the Δ^5 pathway. This, together with the lower catalytic activity of Angora 3 β HSD towards 17-OHPREG subsequently results in less 17-OHPROG intermediate being synthesized which in turn results in reduced cortisol biosynthesis.

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4.2 Influence of 3 β HSD activity on 17-OHPREG metabolism

As 3 β HSD and CYP17 both compete for PREG and 17-OHPREG during adrenal steroidogenesis, variations in 3 β HSD activity would impact the metabolism of both these substrates. However, due to the preliminary nature of the published study, the investigation focused only on the metabolism of PREG and did not consider the role of 3 β HSD on the metabolism of 17-OHPREG specifically. Furthermore, it is known that the metabolism of 17-OHPREG is altered significantly by cytochrome-b₅ (Cyt-b₅), which selectively stimulates the lyase activity of CYP17 resulting in a substantial increase in C19 steroid production (Katagiri et al., 1982; Katagiri et al., 1995; Auchus et al., 1998). Additional studies were therefore performed to investigate the influence of 3 β HSD activity on 17-OHPREG metabolism and the manner in which augmentation of the lyase activity of CYP17 by Cyt-b₅ influences substrate metabolism. In COS-1 cells, Angora and ovine 3 β HSD were each coexpressed with Angora CYP17 in the presence and absence of overexpressed Angora Cyt-b₅ (Goosen et al., 2010). The mammalian expression vector pCI-neo, containing no DNA insert, was added in control assays conducted where Cyt-b₅ was not overexpressed. Each construct was subsequently assayed with 17-OHPREG (1 μ M) as substrate and the relevant steroid metabolites characterized as previously described (Goosen et al., 2010).

The results showed that in the absence of overexpressed Cyt-b₅, cells expressing Angora 3 β HSD produced significantly less 17-OHPROG ($p < 0.01$) compared to cells expressing ovine 3 β HSD (Fig 4.1A). These results correlated with the results obtained for PREG metabolism, suggesting that the reduced activity of Angora 3 β HSD towards 17-OHPREG (Fig 1. in Goosen et al., 2010) significantly alters the competition between 3 β HSD and CYP17 leading to reduced levels of the 17-OHPROG intermediate with a subsequent reduction in cortisol biosynthesis. Thus, these results provided additional evidence that 3 β HSD is a contributing factor in causing hypocortisolism in the South African Angora goat.

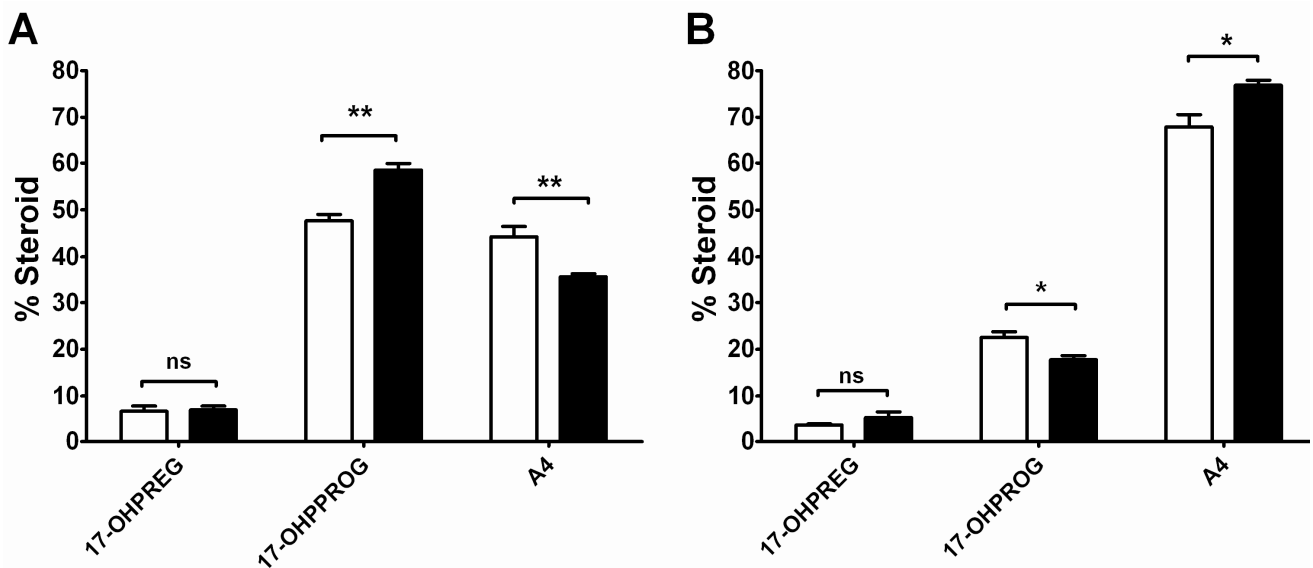


Figure 4.1 Steroid profile of 17-OHPREG (1 μ M) metabolism after 1.5 h by Angora (□) and ovine (■) 3 β HSD coexpressed with Angora CYP17 ACS-. (A) 17-OHPREG metabolism in the absence of overexpressed Cyt-b₅. (B) 17-OHPREG metabolism in the presence of overexpressed Cyt-b₅. The percentage of each steroid metabolite produced relative to total steroid output following the metabolism of 17-OHPREG is depicted. Results are representative of three individual experiments, each performed in triplicate. Individual steroids were compared for each construct using an unpaired *t*-test ($p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **)

It is furthermore known that Cyt-b₅ stimulates the lyase activity of CYP17 selectively, resulting in a substantial increase in the production of C19 steroids. Therefore it was expected that the presence of overexpressed Cyt-b₅ would result in reduced 17-OHPROG biosynthesis and increased A4 synthesis via DHEA. In addition, due to the differences in the catalytic activity between Angora and ovine 3 β HSD (Fig 1, Table 3 in Goosen et al., 2010), it was expected that cells expressing Angora 3 β HSD would produce lower levels of 17-OHPROG when compared to cells expressing ovine 3 β HSD. As expected, the presence of overexpressed Cyt-b₅ substantially increased A4 production at the expense of 17-OHPROG production (Fig 4B). However, the presence of overexpressed Cyt-b₅ caused cells expressing Angora 3 β HSD to produced significantly ($p < 0.05$) higher levels of 17-OHPROG compared to cells expressing ovine 3 β HSD (Fig 4B).

These results sharply contrasted those obtained in the absence of overexpressed Cyt-b₅. In the Angora goat, the substrate for the 17,20 lyase reaction is almost exclusively 17-OHPREG with negligible A4 formation from 17-OHPROG (Storbeck et al., 2007). It is well documented that the presence of overexpressed Cyt-b₅ substantially enhances the lyase activity of CYP17 towards 17-

OHPREG whilst leading to a comparatively small enhancement in activity towards 17-OHPROG. Consequently, the presence of overexpressed Cyt-b₅ would have substantially increased A4 production at the expense of 17-OHPROG production via DHEA. Furthermore, as the lyase activity of CYP17 is augmented equally in cells expressing either Angora or ovine 3 β HSD, the presence of overexpressed Cyt-b₅ could not account for the differences observed in 17-OHPROG production. These results could therefore not be explained using the current model describing the influence of Cyt-b₅ on adrenal steroidogenesis and alluded to the possibility that Cyt-b₅ may affect steroidogenesis in a way other than that which is currently accepted. One possible explanation for the altered metabolism of 17-OHPREG is the specific augmentation of 3 β HSD activity by Cyt-b₅. However, no evidence, other than these findings, is currently available in the literature that would suggest Cyt-b₅ to influence 3 β HSD activity in any way. Nevertheless, differential augmentation of Angora and ovine 3 β HSD activity towards 17-OHPREG by Cyt-b₅ would have a substantial impact on the metabolism of 17-OHPREG which could account for the altered steroid profile observed. This represented an intriguing possibility which necessitated further investigation.

4.3 Discussion and Conclusion

The data presented in this chapter indicate that differences in catalytic activity between Angora and ovine 3 β HSD alter the competition between 3 β HSD and CYP17, leading to a significant alteration in the flux of steroid intermediates through the steroidogenic pathways. This is clearly illustrated by the steroid profile produced by COS-1 cells coexpressing either Angora or ovine 3 β HSD, together with Angora CYP17, following the metabolism of 17-OHPREG. Angora 3 β HSD, in comparison to ovine 3 β HSD, exhibits a lower activity towards 17-OHPREG and a greater activity towards DHEA. Therefore, cells coexpressing Angora 3 β HSD produced significantly greater levels of A4 at the expense of 17-OHPROG following the metabolism of 17-OHPREG (Fig 4.1). However, due to the complexity of the steroid hormone biosynthesis pathway, predictions regarding changes in steroidogenic output in response to variations in 3 β HSD activity are challenging. This is illustrated by the steroidogenic profile produced by COS-1 cells following the metabolism of PREG. Even though cells coexpressing Angora 3 β HSD still produced significantly less 17-OHPROG (Fig 2), correlation between 3 β HSD activity and steroid output is difficult. This is best illustrated by the level of PROG production where cells expressing Angora 3 β HSD produced significantly greater amounts compared to cells expressing ovine 3 β HSD, even though the initial reaction rates of these enzymes towards PREG are similar (Fig 2; Table 2). The precise cause of this phenomenon is as yet uncertain but it may be speculated that variations in

3 β HSD activity downstream may alter the demand for PREG by the Δ^5 pathway, thereby leading to increased 17-OHPREG production at the expense of PROG production in cells expressing ovine 3 β HSD.

Nevertheless, the South African Angora goat has been shown to produce significantly less glucocorticoids precursors compared to the Boer goat and Merino sheep (Engelbrecht and Swart, 2000). Therefore, these data correlate with data obtained *in vivo* in that cells expressing Angora 3 β HSD produced significantly less of the cortisol precursor 17-OHPROG. However, an outright comparison between the steroid production profiles observed in COS-1 cells and that of the adrenal *in vivo* presents numerous challenges. As discussed in chapters 1 and 2, the adrenal cortex is comprised of different factional zones that exhibit variations in steroidogenic enzyme expression which also differ between species. Furthermore, 3 β HSD is unique amongst the steroidogenic enzymes in that it shows dual sub-cellular localization with the degree of distribution between the ER membrane and mitochondrial membrane varying between different tissues and species (Labrie et al., 1992; Simard et al., 1996; Penning, 1997; Payne and Hales, 2004). Therefore, the degree to which 3 β HSD, CYP17 and Cyt-b₅ may be coexpressed in the ER membrane in the adrenal may be vastly different to the ratios in which these enzymes are expressed in the COS-1 cell expression system and may also vary between the Angora and ovine adrenals. To date, no data is available describing the expression profile of 3 β HSD, CYP17 and Cyt-b₅ in the adrenals of the Angora or ovine species. Furthermore, in addition to variations in the relative expression levels, the activity of other steroidogenic enzymes in the steroidogenic network may also differ, resulting in substantial differences in steroidogenic output. The most notable example of this is CYP17 which exhibits significant differences in catalytic activity between these two species, resulting in its implication as the primary cause of hypocortisolism in the South African Angora goat. Thus, the competitive interaction between 3 β HSD and CYP17 and its influence on adrenal steroidogenic output is not only influenced by differences in 3 β HSD activity but also CYP17 and the ratio in which these enzymes are coexpressed in the ER membrane. Therefore, additional research is needed to determine the relative expression levels of these key steroidogenic enzymes *in vivo* in order to better understand how variations in 3 β HSD activity may influence adrenal steroidogenesis.

Nevertheless, the data presented in this chapter clearly indicate that differences in catalytic activity between Angora and ovine 3 β HSD alter the competition between 3 β HSD and CYP17, leading to a significant alteration in the flux of steroid intermediates through the steroidogenic pathways. The data revealed a significant reduction in the biosynthesis of the cortisol precursor 17-OHPROG by cells expressing Angora 3 β HSD following the metabolism of PREG and 17-OHPREG, which correlated

with results obtained *in vivo* and implicated 3 β HSD as an additional factor in causing hypocortisolism in the South African Angora goat.

In addition, the presence of overexpressed Cyt-b₅ was found to significantly alter the competition between 3 β HSD and CYP17 for 17-OHPREG leading to an altered steroidogenic profile. This profile could not be explained using the current model describing the influence of Cyt-b₅ on adrenal steroidogenesis, eluding to the possibility that Cyt-b₅ may specifically augment 3 β HSD activity.

The following chapter therefore describes a study which aimed to determine whether such an augmentation exists, and if so, whether it is specific to 3 β HSD or an artifact of the COS-1 cell expression system.

CHAPTER 5

CYTOCHROME b_5 AUGMENTS 3β -HYDROXYSTEROID DEHYDROGENASE/ Δ^5 - Δ^4 ISOMERASE ACTIVITY

5.1 Introduction

The results presented in the previous chapter allude to the possibility that Cyt- b_5 might augment 3β HSD activity. However, no experimental data were available describing the influence of Cyt- b_5 on 3β HSD activity or any other member of the hydroxysteroid dehydrogenases. Therefore, the aim of this study was to determine whether augmentation of 3β HSD activity by Cyt- b_5 existed, and if so, whether such augmentation was specific to 3β HSD. To achieve this aim Cyt- b_5 was coexpressed with both Caprine and ovine 3β HSD in COS-1 cells and the catalytic activity assayed towards PREG, 17-OHPREG and DHEA. In addition, ovine adrenal microsomes were prepared and the 3β HSD activity was assayed in the presence and absence of purified ovine liver Cyt- b_5 and anti-Cyt- b_5 IgG. The results were published in the Journal of Steroid Biochemistry and Molecular Biology and are presented in the article included in this chapter (Goosen et al., 2011). [Reprinted from the Journal of Steroid Biochemistry and Molecular Biology, 127, Pierre Goosen, Karl-Heinz Storbeck, Amanda C. Swart, Riaan Conradie and Pieter Swart, Cytochrome b_5 augments 3β -Hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase activity, 238-247, 2011, with permission from Elsevier, license no. 3000030738192]

As no data were previously available describing the influence of Cyt- b_5 on 3β HSD activity, this chapter will provide a brief background on Cyt- b_5 in the context of its known involvement in various cytochrome P450 catalyzed reactions.

5.2 Cytochrome b_5

Cyt- b_5 is a small ubiquitous electron transfer hemoprotein known to participate in a variety of biochemical reactions in various tissue types. These include: the reduction of methemoglobin to hemoglobin (Hultguist and Passon, 1971; Sannes and Hultquist, 1978), fatty acid desaturation and elongation (Keyes and Cinti, 1980; Okayasu et al., 1981), plasmalogen and cholesterol biosynthesis (Paltauf et al., 1974; Fukushima et al., 1981) and the biosynthesis of N-glycolylneuraminic acid (Kawano et al., 1994). In addition, Cyt- b_5 has also been shown to affect the substrate metabolism of

various cytochromes P450 involved in xenobiotic metabolism and steroidogenesis (Vergères and Waskell, 1995; Porter, 2002; Yamazaki et al., 2002; Schenkman and Jansson, 2003 and references therein).

In mammals, Cyt-b₅ exists as both a soluble cytosolic protein and an amphipathic membrane-bound protein. The soluble cytosolic form consists of 98 amino acids and is expressed primarily in erythrocytes. The amphipathic membrane-bound form is divided into two species, depending on primary structure and sub-cellular localization. The first is localized to the endoplasmic reticulum and consists of a 134 amino acids whilst the second is localized primarily to the outer mitochondrial membrane and consists of 146 amino acids (Ito, 1980; Lederer et al., 1983; Giordano and Steggles, 1991; Giordano et al., 1993; Li et al., 1995; Kuroda et al., 1998; Pandey and Miller, 2005). These isoforms are widely expressed and have been detected in a variety of different tissues, including the adrenals, gonads, liver, kidney, fat, spleen, lung and brain (Garfinkel, 1963; Giordano and Steggles, 1991; 1993). In humans, Cyt-b₅ is encoded by two genes which undergo alternative splicing to produce each respective isoform. The first gene is located on chromosome 18q23 and consists of six exons. The soluble cytosolic form is encoded by exons 1-4 whilst the membrane-bound 134 amino acid form is encoded by exons 1-3 and 5-6. The second gene is located on chromosome 16q22.1 and consists of five exons that encode for the membrane-bound 146 amino acid mitochondrial form (Cristiano et al., 1993; Giordano and Steggles., 1991; 1993; Giordano et al., 1993; Kuroda et al., 1998; Pandey and Miller, 2005).

The 134 amino acid form of Cyt-b₅ is the only form known to significantly affect adrenal steroidogenesis and is also the most abundant form present in the adrenal (Porter, 2002; Schenkman and Jansson, 2003; Pandey and Miller, 2005; Omura, 2010 and reference therein). Therefore, the remainder of this chapter will focus on the 134 amino acid microsomal form, its structure and the mechanisms through which it affects cytochrome P450 catalyzed reactions.

5.2.1 Structure

The microsomal form of Cyt-b₅ is a small acidic membrane-bound protein cylindrical in shape. The protein consists of six α -helices and five β -strands which are folded into two domains. The larger hydrophilic head domain consists of the cytosolic heme-containing amino-terminal whilst the smaller hydrophobic tail domain consists of a membrane-binding carboxyl portion of 14-18 residues. These two domains are connected via a Pro containing hinge region of approximately seven residues followed

by seven polar residues at the end of the carboxyl-terminal (Mathews, 1985; Ozols, 1989; Lu et al., 1995; Schenkman and Jansson, 2003).

Protease can cleave before or after the hinge region releasing the soluble catalytic domain from the membrane. However, studies have shown that proteolytically solubilised Cyt-b₅ is unable to modify the activities of microsomal P450s, suggesting that only the membrane-bound form has the ability to significantly affect steroidogenesis (Imai and Sato, 1977; Sygiyama et al., 1980; Schenkman and Jansson, 2003).

The heme moiety of Cyt-b₅ is contained in a cleft within the cytosolic domain between four α -helices with the edge of the heme exposed to the environment. This region forms a hydrophobic pocket in which the heme iron is coordinated by two highly conserved His residues at positions 44 and 68 (Fig 5.1). The fifth and sixth coordinates of the heme iron interact with these His residues, thereby preventing its direct interaction with molecular oxygen. Nevertheless, Cyt-b₅ is still able to participate in electron transfer reactions as an intermediate between reductases and oxidative enzymes. For example, Cyt-b₅ can accept electrons from P450 oxidoreductase or cytochrome b₅ reductase and transfer them to a number of cytochromes P450 (Hildebrandt and Estabrook, 1971; Oshino et al., 1971; Schenkman and Jansson, 2003).

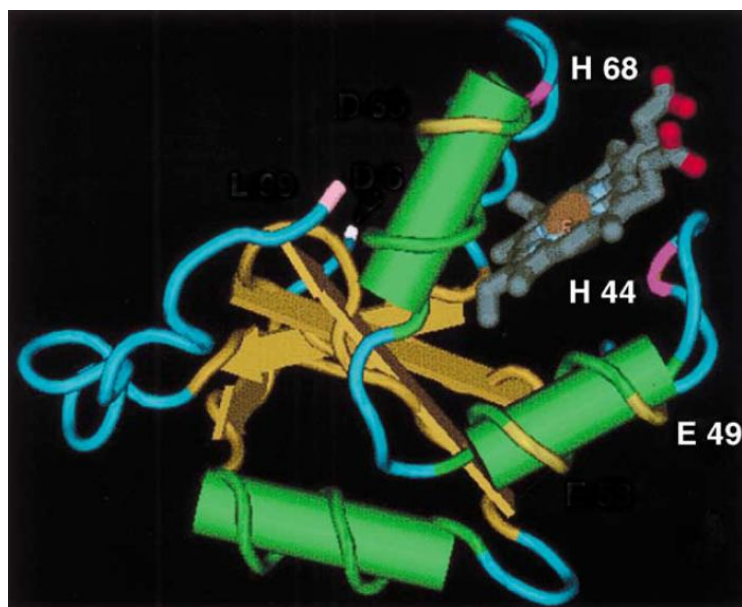


Figure 5.1 3-D model of the heme domain of Cyt-b₅ based on the NMR structure. The heme moiety, shown in grey, is positioned in a cleft formed by various α -helices. The two His residues, His44 and His68, responsible for coordinating the heme moiety are shown in pink. Glu49, implicated in charge pairing interactions, is also shown. Reproduced from Schenkman and Jansson (2003).

The sequence of the catalytic domain is highly conserved through out all species whilst the entire protein shows over 80% identity in mammals (Ozols, 1989). As mentioned above, Cyt-b₅ is largely acidic with 23 of the 134 amino acids being either Glu or Asp. A number of these acidic residues are located around the heme edge (e.g. Glu49, Glu53, Asp65) and protrude into the plane of the solvent. These acidic residues are well conserved and have been implicated in charge pairing interactions (Strittmatter et al., 1990; Qian et al., 1998; Schenkman and Jansson, 2003). Indeed, it has been suggested that residues Glu48 and Glu49 may form part of an allosteric binding site responsible for mediating the stimulation of CYP17 lyase activity (Naffin-Olivos and Auchus, 2006).

5.2.2 Mechanism of action

Cyt-b₅ has long been known to augment various cytochrome P450 catalyzed reactions, however, the mechanism of stimulation has remained controversial (Porter, 2002; Schenkman and Jansson, 2003; Omura, 2010; McLaughlin et al., 2010). Several factors contribute to the controversy regarding the nature of this mechanism, including findings which indicate that Cyt-b₅ may enhance or inhibit P450 catalysis depending on the specific enzyme and substrate involved (Gruenke et al., 1995; Zhang et al., 2007). In addition, conflicting reports that apo-b₅ (Cyt-b₅ void of heme) can stimulate P450 catalysis have added to this controversy (Guryev et al., 2001; Yamazaki et al., 2001; Porter, 2002; Schenkman and Jansson, 2003; Akhtar et al., 2005). In order to account for these observations two general mechanisms were proposed through which Cyt-b₅ stimulates P450 catalysis. The first mechanism requires the participation of Cyt-b₅ in the P450 catalytic cycle as an electron transfer protein, whilst in the second, Cyt-b₅ acts as an allosteric modulator without directly participating in electron transfer. The following section will briefly discuss these general mechanisms.

5.2.2.1 Electron transfer

As described in chapter 2, cytochrome P450 enzymes require the input of two electrons from an external electron donor to perform their catalytic function. According to the first general mechanistic model, Cyt-b₅ may augment P450 catalyzed reactions by directly participating in electron transfer. One model suggests that during the reductive stages of the P450 catalytic cycle, Cyt-b₅ provides the second electron required for the completion of the cycle instead of the conventional reductase (Fig 5.2) (Hildebrandt and Estabrook, 1971; Schenkman and Jansson, 2003; Akhtar et al., 2005).

Previous studies have found that P450 oxidoreductase (POR) can readily reduce Cyt-b₅ without requiring NADH or cytochrome b₅ reductase (Enoch and Strittmatter, 1979). Furthermore, although the reduction of ferric P450 by Cyt-b₅ is thermodynamically unfavorable the reduction of oxyferrous P450 (one electron reduced form) by Cyt-b₅ can occur readily, allowing Cyt-b₅ to contribute the second electron to the catalytic cycle (Guengerich, 1983; Porter, 2002). Therefore, according to this model, Cyt-b₅ acts as an alternative redox partner during the transfer of the second electron. This is thought to result in the faster input of the second electron, reducing the spontaneous decay of the oxycytochrome P450 complex, allowing more product to form (Hildebrandt and Estabrook, 1971; Schenkman and Jansson, 2003).

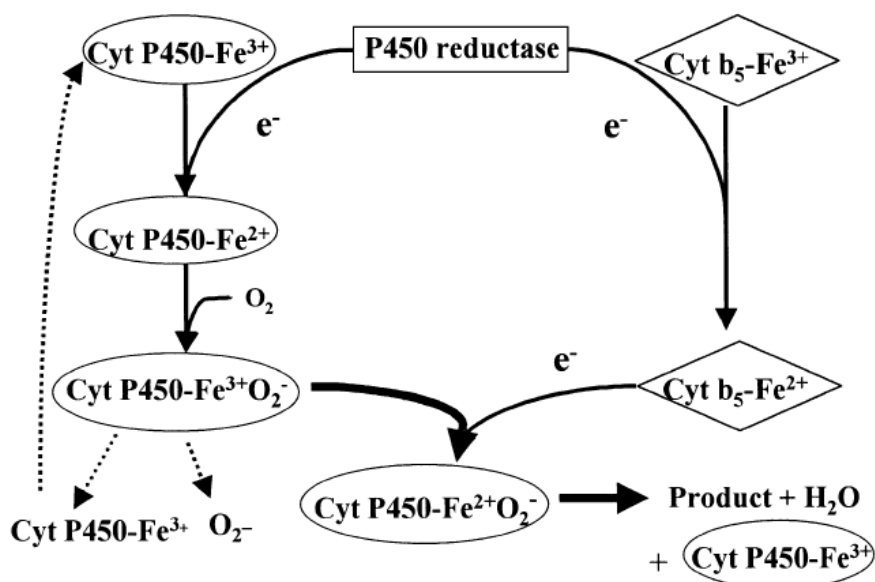


Figure 5.2 Schematic representation of the proposed mechanism through which Cyt-b₅ provides the second electron during the P450 catalytic cycle. The dotted line indicates the reduced decay of the oxycytochrome P450 complex, allowing more product to form. Reproduced from Schenkman and Jansson (2003).

In the case of cytochrome P450 2B4 (CYP2B4) it has been demonstrated that Cyt-b₅ and POR reduce the P450 at a similar rate but that catalysis nevertheless occurs more rapidly in the presence of Cyt-b₅ (Zang et al., 2003; Zang et al., 2007). In addition, Cyt-b₅ has also been demonstrated to compete with POR for non-identical but overlapping binding sites on the proximal surface of CYP2B4 (Bridges et al., 1998; Zang et al., 2003). Therefore, a second electron transfer model has been proposed that suggests that both the electron donating ability and effector (allosteric) function of Cyt-b₅ may operate

to alter CYP2B4 catalysis. According to this hypothesis, during the transfer of the second electron, Cyt-b₅ binds to CYP2B4 causing conformational changes in the active site, allowing the oxyferrous specie to be formed more rapidly in the presence of Cyt-b₅ compared to the reductase. During low molar ratios of Cyt-b₅:POR, P450 catalysis is stimulated resulting in enhanced substrate metabolism. However, high molar ratios of Cyt-b₅:POR inhibit P450 catalysis by binding to the proximal binding site. This prevents the binding of POR, thereby preventing the transfer of the first electron and the initiation of the P450 catalytic cycle. The exact molecular mechanism through which Cyt-b₅ stimulates CYP2B4 catalysis is however still unclear (Bridges et al., 1998; Zang et al., 2003, 2007).

5.2.2.2 *Allosteric*

According to the second general mechanistic model, Cyt-b₅ modulates P450 catalysis through an allosteric mechanism independent of direct electron transfer.

The first evidence of an allosteric role for Cyt-b₅ during the stimulation of P450 catalyzed reactions came from studies in which the heme moiety was replaced with manganese-protoporphyrin IX. Even though this mutant protein is unable to accept or transfer electrons it retained the ability to decrease the K_m values of CYP2B4 and CYP1A2 towards several substrates (Morgan and Coon, 1984). Further evidence for an allosteric mechanism came from the observation that apo-b₅ stimulated cytochrome P450 3A4 (CYP3A4) catalysis to the same extent as holo-b₅ (normal wild-type Cyt-b₅) (Yamazaki et al., 1996). However, this was challenged by Guryev et al (2001) who showed that the addition of a heme scavenger, apo-myoglobin, removed the stimulatory effects of apo-b₅. The authors concluded that apo-b₅ is only stimulatory after transformation to holo-b₅ through the uptake of excess heme from the preparation. This was countered by Yamazaki et al (2001) who showed that the amount of excess heme in the preparation is less than that which could account for the stimulation observed with apo-b₅. In addition, the authors also showed a lack of inhibition of apo-b₅ stimulation by apo-myoglobin, supporting an allosteric role for Cyt-b₅. Since these observations, allosteric stimulation by Cyt-b₅ has been documented for a number of drug-metabolizing P450s including: CYP2C9, 4A7, 2A6, 2B6, 2C8, 2C19 and 3A5 (Yamazaki et al., 2002) Furthermore, a growing body of evidence also indicates that Cyt-b₅ plays an important allosteric role in regulating the 17,20-lyase activity of CYP17 during steroidogenesis (Auchus et al., 1998; Lee-Robichaud et al., 1998; Geller et al., 1999; Akhtar et al., 2011).

Using a reconstituted assay system, Auchus et al (1998) showed that CYP17 lyase activity could be stimulated by varying the molar ratios of Cyt-b₅:CYP17 whilst maintaining a fixed POR concentration. Maximal stimulation was observed when the Cyt-b₅:CYP17 ratio was adjusted between 10:1 and 30:1. However, the stimulatory effect of Cyt-b₅ was substantially decreased in the presence of excess Cyt-b₅, which the authors attributed to electron scavenging by Cyt-b₅ from POR. This was substantiated by using cytochrome c as an alternative electron acceptor, which showed a similar pattern of inhibition at equimolar amounts to Cyt-b₅. Furthermore, apo-b₅ was shown to exhibit a similar stimulatory profile as holo-b₅, however, apo-b₅ did not exhibit the same inhibitory effect when the Cyt-b₅:CYP17 ratio was adjusted beyond the optimal range (Auchus et al., 1998). Lee-Robichaud et al (1998) reported similar observations with Mn²⁺-substituted Cyt-b₅, suggesting an allosteric function for Cyt-b₅ during the stimulation of CYP17 lyase activity.

The exact molecular mechanism through which Cyt-b₅ stimulates CYP17 lyase activity is still uncertain. However, Auchus et al (1998) hypothesized that Cyt-b₅ modifies the CYP17·POR complex allosterically, thereby facilitating more efficient electron transfer during catalysis (Fig 5.3). According to this model, NADPH transfers two electrons to the FAD domain of POR, which passes the electrons to the FMN moiety. As the FMN and FAD domains of POR are connected by a hinge region, the FMN domain must rotate $\approx 90^\circ$ to dock with the redox-partner binding site of CYP17. This interaction of POR with CYP17 is adequate to support 17 α -hydroxylation, however, it rarely adopts the geometry that satisfies the more stringent conformational restrictions required for the 17,20-lyase reaction. It is hypothesized that Cyt-b₅ acts as an allosteric effector to facilitate the interaction between POR and CYP17 in such a way that satisfies the conformational requirements for the 17,20-lyase reaction. This facilitates more efficient electron transfer which subsequently increases the rate of catalysis and product formation (Auchus et al., 1998) (Fig 5.3).

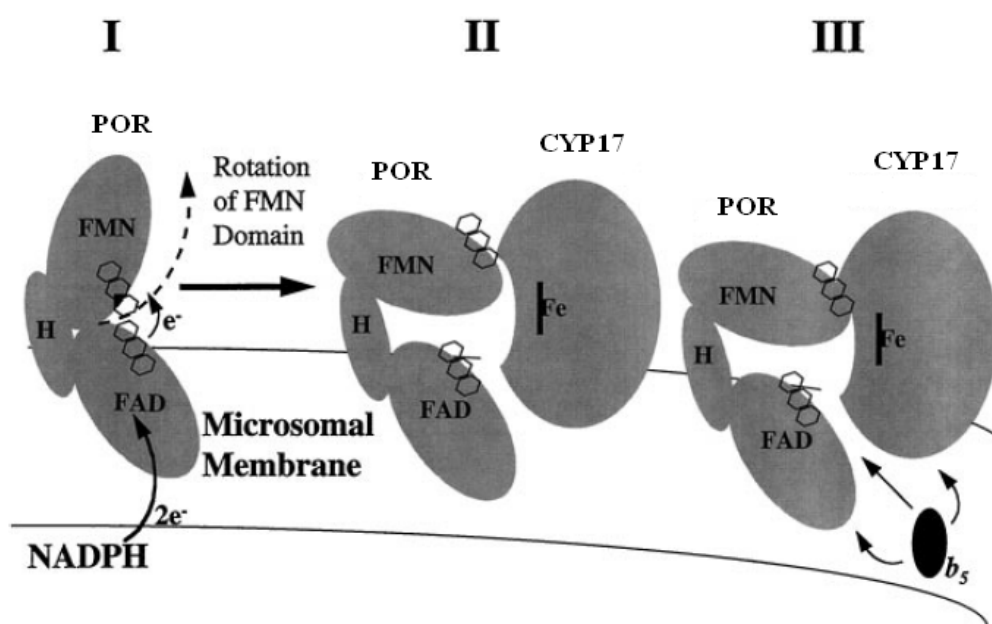


Figure 5.3 Proposed function of Cyt-b₅ in CYP17 catalysis. **(I)** NADPH transfers two electrons to POR. **(II)** FMN domain of POR rotates to dock with the redox-partner binding site on CYP17, resulting in the 17 α -hydroxylation of the steroid substrate. **(III)** Cyt-b₅ acts as an allosteric effector to optimize the geometry required for the 17,20-lyase reaction. Reproduced from Auchus et al (1998).

It is generally believed that cytochromes P450 interact with their redox partners via ionic interactions. Yamazaki et al (2002) demonstrated that positive surface charges on P450s form ionic interactions with clusters of surface negative charges on their redox partners. In the case of CYP17, mutant enzymes containing either an Arg347His or Arg358Gln mutation showed $\approx 5\%$ lyase activity when expressed in COS-1 cells in the presence of Cyt-b₅ (Geller et al., 1999). In addition, substitution of these Arg residues with Lys residues did not influence the hydroxylase or lyase activity of CYP17. Furthermore, substitution of Arg449 with an Ala residue abolishes Cyt-b₅ dependant 17,20-lyase activity. These results indicate that the interaction between CYP17 and Cyt-b₅ involves cationic residues on CYP17, in particular Arg347, Arg358 and possibly Arg349. The anionic residues on Cyt-b₅ which pare with the aforementioned cationic residues on CYP17 are not fully known (Lee-Robichaud et al., 1998; 1999; 2004; Akhtar et al., 2011). However, it has been shown that substitution of Glu48 and Glu49 with Gly residues resulted in mutant proteins which exhibit a decreased ability to stimulate CYP17 lyase activity (Naffin-Olivos and Auchus, 2006).

Therefore, as Cyt-b₅ is known to augment various enzyme catalyzed reactions allosterically and 3 β HSD does not require electrons from an external electron donor to perform its catalytic function we hypothesized that Cyt-b₅ augmented 3 β HSD activity via an allosteric mechanism.

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journal homepage: www.elsevier.com/locate/jsbmbCytochrome b₅ augments 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase activity

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ABSTRACT

During adrenal steroidogenesis the competition between 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β HSD) and cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17A1) for Δ^5 steroid intermediates greatly influences steroidogenic output. Cytochrome-b₅ (Cyt-b₅), a small electron transfer hemoprotein, known to augment the lyase activity of CYP17A1, has been shown to alter the steroidogenic outcome of this competition. In this study, the influence of Cyt-b₅ on 3 β HSD activity was investigated. In COS-1 cells, Cyt-b₅ was shown to significantly increase the activity of both caprine and ovine 3 β HSD towards pregnenolone, 17-OH pregnenolone and dehydroepiandrosterone in a substrate and species specific manner. Furthermore, kinetic studies revealed Cyt-b₅ to have no influence on the K_m values while significantly increasing the V_{max} values of ovine 3 β HSD for all its respective substrates. In addition, the activity of ovine 3 β HSD in microsomal preparations was significantly influenced by the addition of either purified Cyt-b₅ or anti-Cyt-b₅ IgG. The results presented in this study indicate that Cyt-b₅ augments 3 β HSD activity and represents the first documentation of such augmentation in any species.

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1. Introduction

Hydroxysteroid dehydrogenases play pivotal roles in the biosynthesis and inactivation of steroid hormones. In steroidogenic tissue, members in this class catalyze the biosynthesis of steroid hormones while they convert potent steroid hormones to inactive metabolites in peripheral tissue [1].

During the biosynthesis of steroid hormones in the adrenal cortex, 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β HSD) plays a central role in the production of mineralocorticoids, glucocorticoids and C19 steroids [2]. 3 β HSD catalyzes the conversion of the Δ^5 steroids, pregnenolone (PREG), 17-hydroxypregnenolone (17-OHPREG) and dehydroepiandrosterone (DHEA) to the corresponding Δ^4 steroids, progesterone (PROG), 17-hydroxyprogesterone (17-OHPROG) and androstenedione (A4) through a sequential two-step reaction [3,4]. In the first dehydrogenation reaction, NAD⁺ is reduced to NADH with the formation of a Δ^5 - Δ^4 keto steroid intermediate which, together with NADH, remains bound to the enzyme. The bound NADH induces conformational changes in the enzyme resulting in the isomerization of

the bound intermediate to form the corresponding Δ^4 steroid product [4,5]. According to the model proposed by Thomas et al. [4], the dehydrogenase and isomerase domains of the enzyme are linked by a shared coenzyme domain that functions as both the binding site for NAD⁺ during the dehydrogenase reaction and for the reduced NADH, with the latter subsequently acting as an allosteric activator of the isomerase reaction.

During the synthesis of steroid hormones, 3 β HSD competes with cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17A1) for Δ^5 -steroid intermediates. CYP17A1 catalyzes two distinct reactions, the 17 α -hydroxylation of PREG and PROG yielding 17-OHPREG and 17-OHPROG, and the subsequent 17,20 lyase reaction resulting in the cleavage of the C17,20 bond yielding DHEA and A4, respectively [6–8]. Due to the competition between 3 β HSD and CYP17A1 for PREG and 17-OHPREG, the ratio of the enzyme activities and substrate specificities are crucial in determining the flux of steroid intermediates through the steroidogenic pathways [9–12]. This competition is significantly altered by cytochrome-b₅ (Cyt-b₅), which selectively stimulates the lyase activity of CYP17A1 resulting in a substantial increase in C19 steroid production [13,14].

Cyt-b₅ is a small ubiquitous electron-transfer hemoprotein. The microsomal form of Cyt-b₅ expressed in steroidogenic tissue consists of two domains: a larger globular head domain, which contains the heme moiety, and a smaller hydrophobic membrane anchoring tail domain. The two domains are connected by a proline containing hinge region [15,16]. To date, studies investigating the influence of Cyt-b₅ on steroidogenesis have focused primarily on

Abbreviations: apo-b₅, wild type cytochrome-b₅ void of heme; POR, P450 oxidoreductase; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; *holo*-b₅, wild type cytochrome-b₅; *trunc*-b₅, truncated wild type cytochrome-b₅.

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the stimulation of CYP17A1 lyase activity as none of the other steroidogenic cytochromes P450 have been shown to be influenced by Cyt-b₅. In addition, no data is available describing the influence of Cyt-b₅ on 3 β HSD.

Since the augmentation of 3 β HSD activity by Cyt-b₅ would contribute towards the alteration in steroidogenic output as is observed for CYP17A1 in the presence of Cyt-b₅, the potential influence of Cyt-b₅ on 3 β HSD activity was investigated. In this study, Cyt-b₅ was co-expressed with both caprine and ovine 3 β HSD in nonsteroidogenic COS-1 cells and the catalytic activities towards PREG, 17-OHPREG and DHEA assayed in the absence of other steroidogenic enzymes. In addition, ovine adrenal microsomes were prepared and 3 β HSD activity assayed in the presence and absence of purified Cyt-b₅ and anti-Cyt-b₅ IgG.

2. Materials and methods

2.1. Materials

Plasmid vectors, Gene Tailor™ site-directed mutagenesis system and MultiSite Gateway® Pro cloning system were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Nucleobond® AX plasmid purification kits were supplied by Macherey-Nagel (Duren, Germany) and Wizard® Plus SV Minipreps DNA Purification kits were purchased from Promega Biotech (Madison, WI, USA). COS-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Mirus TransIT®-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Fetal calf serum and bacterial culture medium were purchased from Highveld Biological (Lyndhurst, SA) and Difco Laboratories (Detroit, MI, USA), respectively. Penicillin–streptomycin, trypsin–EDTA and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). [1,2,6,7-³H]-DHEA and [7-³H]-PREG were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). PREG, 17-OHPREG, DHEA, Dulbecco's modified Eagle's medium (DMEM) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest quality and obtained from reputable scientific supply houses.

2.2. Preparation of plasmid constructs

Angora goat/caprine (GenBank accession no. EF524065), ovine 3 β HSD (GenBank accession no. FJ007375) and caprine Cyt-b₅ (GenBank accession no. EF524066) were previously cloned into the pcDNA3.2/V5/GW/D-TOPO® vector (Invitrogen Life Technologies, Carlsbad, CA, USA) as described by Storbeck et al. [17] and Goosen et al. [18].

Apo- (wild type Cyt-b₅ void of heme) and *trunc-* (truncated wild type Cyt-b₅) forms of caprine Cyt-b₅ were prepared using the Gene Tailor™ site-directed mutagenesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers used to create *apo-b₅*, which contained a H68A mutation [19], were: sense primer, 5'-AAA ACT TTG AGG ACG TTG GAG CCT CTA CAG ATG CT-3'; antisense primer, 5'-TCC AAC GTC CTC AAA GTT TTC AGT GGC ATC-3'.

Trunc-b₅ was created by inserting a stop codon at position 270 terminating translation of 45 amino acid residues at the C-terminus of *holo-b₅* (wild type Cyt-b₅). The primers used were: sense primer, 5'-GCT GCA CCC GGA TGA CAG ATG AAA GAT AAC CA-3'; antisense primer, 5'-ATC TGT CAT CCG GGT GCA GCT CCC CAA TGA-3'. The mutations were subsequently confirmed by direct DNA sequence

analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Johannesburg, RSA).

Ovine 3 β HSD-CFP (Cyan Fluorescent Protein) and *holo-b₅*-YFP (Yellow Fluorescent Protein) plasmid constructs were prepared using the MultiSite Gateway® Pro cloning system (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The primers used to amplify ovine 3 β HSD from the pcDNA3.2/V5/GW/D-TOPO® vector and CFP from the Cer-5-Ven vector during the construction of the 3 β HSD-CFP chimeric construct were: sense primer 3 β HSD-attB1, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG CGG GCT GGA GCT GCC TG-3'; antisense primer 3 β HSD-attB5r, 5'-GGG GAC AAC TTT TGT ATA CAA AGT TGT ATG AAT TTT GGT TTT CAG GGT-3' and sense primer CFP-attB5, 5'-GGG GAC AAC TTT GTA TAC AAA AGT TGC GAT GGT GAG CAA GGG CGA GG-3'; antisense primer CFP-attB2r, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCC GGA CTT GTA CAG CTC-3'. The primers used to amplify *holo-b₅* from the pcDNA3.2/V5/GW/D-TOPO® vector and YFP from the Cer-5-Ven vector during the construction of the *holo-b₅*-YFP chimeric construct were: sense primer *holo-b₅*-attB5, 5'-GGG GAC AAC TTT GTA TAC AAA AGT TGC GTC GCT GAG TTA AGA AAT GGC C-3'; antisense primer *holo-b₅*-attB2r, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTC CCT GGA CCA AAG CAG-3' and sense primer YFP-attB1, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA GAT CCG CTA GCG CTA C-3'; antisense primer YFP-attB5r, 5'-GGG GAC AAC TTT TGT ATA CAA AGT TGT TCC GGA CTT GTA CAG CTC-3'.

2.3. Caprine and ovine 3 β HSD enzyme activity assay in transiently transfected COS-1 cells

COS-1 cells were grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin and 0.12% NaHCO₃. Cells were plated into 12-well plates with each well containing 1.0×10^5 cells in 1 ml, 24 h prior to transfection. The cells were transiently cotransfected with DNA plasmid constructs, 1 μ g, using TransIT®-LT1 transfection reagent (Mirus), according to manufacturer's instructions.

PREG, 17-OHPREG and DHEA metabolism were assayed in cells cotransfected with either caprine or ovine 3 β HSD (0.5 μ g) and *holo-b₅* (0.5 μ g). Control transfection reactions were performed using either caprine or ovine 3 β HSD (0.5 μ g) and the mammalian expression vector pCI-neo (0.5 μ g) (Promega, Madison, WI, USA) which contained no DNA insert. PREG metabolism was also assayed in cells in which ovine 3 β HSD was cotransfected with equal amounts of either *holo-b₅*, *trunc-b₅*, pCI-neo or human 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2) [20]. The influence of Cyt-b₅ electron transfer on PREG metabolism by 3 β HSD was assayed in cells cotransfected with either *holo-b₅* or *apo-b₅* (0.2–0.8 μ g) and ovine 3 β HSD (0.2 μ g) in ratios ranging from 1:1 to 1:4 with pCI-neo added to keep the amount of DNA constant.

Enzyme activity was assayed after 72 h by adding appropriate steroid substrates – PREG and [³H]PREG; 17-OHPREG; DHEA and [³H]DHEA. At specific time intervals aliquots were removed from the assay mixtures, 50 μ l from the PREG and DHEA conversion assays and 500 μ l from the 17-OHPREG assay. The steroid metabolites were subsequently extracted by liquid–liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 15 min and centrifuged at $500 \times g$ for 5 min. The water phase was aspirated off, and the dichloromethane phase transferred to a clean test tube and dried under N₂. The dried steroid residue was redissolved in 120 μ l methanol prior to HPLC or UPLC–MS analysis. After completion of each experiment, the cells were washed and collected in phosphate buffer (0.1 M, pH 7.4). The cells were homogenized with a small glass homogenizer and the total protein content of the homogenate was determined using

the Pierce BCA™ protein assay kit according to the manufacturer's instructions.

2.4. Expression and visualization of 3 β HSD-CFP and holo-b₅-YFP fusion proteins in transiently transfected COS-1 cells

COS-1 cells were grown as described in Section 2.3. Cells were plated into 6-well plates (2.0×10^5 cells/well) with each well containing a small glass cover slip, 24 h prior to transfection. The cells were transiently cotransfected with DNA plasmid constructs, 2 μ g, using TransIT®-LT1 transfection reagent (Mirus), according to manufacturer's instructions. The level of fluorescence of each fusion protein was investigated in cells cotransfected with either 3 β HSD-CFP (1 μ g) and holo-b₅-YFP (1 μ g) or 3 β HSD-CFP (0.4 μ g) and holo-b₅-YFP (0.4–1.6 μ g) in ratios ranging from 1:1 to 1:4 with pCI-neo added to keep the amount of DNA constant.

Forty-eight hours post-transfection the cells were washed with methanol (–20 °C) and fixed by incubation in methanol (1 ml) for 20 min at –20 °C. Following fixation the cells were washed three times with phosphate buffer containing 0.2% bovine serum albumin and mounted on individual object slides.

The cells were subsequently analyzed for fluorescence using an Olympus CellR system attached to an IX 81 inverted fluorescence microscope (Olympus Corp.) equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). The light source was a 150 W Xenon lamp, part of an MT20 excitation system. Cells were observed with a 60 \times oil immersion objective and the Cell® imaging software was used for image acquisition and analysis. The YFP filter set excited at S500/20x (Chroma) and emission was detected at S535/30m, whereas the CFP filter set excited at S430/25x and emission was detected at S470/30m.

2.5. Preparation of adrenal microsomes

Ovine adrenals were obtained at a local abattoir and immediately stored on ice. All subsequent procedures were performed at 4 °C. Microsomes were prepared according to the differential centrifugation method of Yang and Cederbaum [21]. Briefly, following the removal of the capsule and excess fat, fresh adrenals were washed with a 15 mM KCl solution. The adrenal tissue was homogenized in three volumes 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 0.25 M sucrose and centrifuged for 15 min at $500 \times g$. The supernatant was centrifuged at $12\,000 \times g$ for 15 min. A 50% (w/v) PEG 8000 solution was added while stirring to the postmitochondrial supernatant to a final concentration of 8.5%. The mixture was stirred for 10 min and centrifuged at $13\,000 \times g$ for 20 min. The microsomal pellet was homogenized in 200 ml 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl and 1.0 mM EDTA. A 50% (w/v) PEG 8000 solution was again added to the suspension to a final concentration of 8.5% and stirred for 10 min at 4 °C, followed by centrifugation at $13\,000 \times g$ for 20 min. This procedure was repeated twice until the supernatant was clear. The final microsomal pellet was re-suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 0.25 mM sucrose and stored at –80 °C. The total protein content of the preparation was determined using a Pierce BCA™ protein assay kit according to manufacturer's instructions. Finally, the P450 and Cyt-b₅ content of the microsomal preparation was determined spectrophotometrically, as previously described [22,23].

2.6. Isolation and purification of ovine Cyt-b₅

Ovine liver was obtained at a local abattoir and immediately stored on ice. Liver microsomes were prepared according to the differential centrifugation method of Yang and Cederbaum [21]. Cyt-b₅ was subsequently purified from the microsomal

preparation as described by Swart et al. [10]. Briefly, the microsomal fraction was loaded onto a DEAE-cellulose column (3.2×20 cm) equilibrated with 80 mM Tris acetate, 1 mM EDTA, 2% Triton X-100, pH 8.1. Cyt-b₅ reductase was eluted with 0.1 M Tris acetate, 1 mM EDTA, 2% Triton X-100, pH 8.1 after which a linear gradient from 0 to 50 mM NaSCN was run over 3 bed volumes in 10 mM Tris acetate, 1 mM EDTA, 0.1% deoxycholate, 0.2% Triton X-100, pH 8.1. Cyt-b₅ was subsequently eluted with 90 mM NaSCN in 10 mM Tris acetate buffer and dialyzed overnight against ten volumes 10 mM Tris acetate buffer. Following dialysis, the Cyt-b₅ solution was applied to a DEAE-cellulose column (2×20 cm) equilibrated with 10 mM Tris acetate, 1 mM EDTA, 0.2% Triton X-100, 0.05% deoxycholate, pH 8.1. A linear NaSCN gradient from 0 to 50 mM in equilibration buffer was followed by a linear NaSCN gradient from 50 mM to 90 mM to elute Cyt-b₅. The Cyt-b₅ solution was subsequently dialyzed overnight against 10 volumes 10 mM Tris acetate, 0.1 mM EDTA, pH 8.1. Following dialysis, the solution was applied to a DEAE-cellulose column (1×5 cm) equilibrated with 10 mM Tris acetate, 0.1 mM EDTA, pH 8.1. Finally, Cyt-b₅ was eluted with 10 mM Tris acetate, 0.1 mM EDTA, 0.25% sodium deoxycholate, pH 8.1 and dialyzed overnight against ten volumes of elution buffer and the resulting Cyt-b₅ solution stored at –80 °C. The Cyt-b₅ content was determined spectrophotometrically as previously described [23]. The total protein content of the solution was determined using a Pierce BCA™ protein assay kit according to manufacturer's instructions.

2.7. Microsomal 3 β HSD enzyme activity assay

3 β HSD enzyme activity was assayed in ovine adrenal microsomes as previously described [10]. Briefly, incubations were performed in a water bath at 37 °C in a total volume of 500 μ l. The reaction mixture contained microsomal preparation (0.35 μ M P450) and was carried out in 50 mM Tris buffer containing 1% (m/v) bovine serum albumin and 50 mM NaCl. Following a 5 min pre-incubation of the reaction mixture at 37 °C with either PREG and [³H]PREG; 17-OHPREG or DHEA and [³H]DHEA as substrates, the reaction was initiated by the addition of 0.5 mM NAD⁺. Aliquots (50 μ l) were removed from the assay mixture at specific time intervals and the steroid metabolites subsequently extracted by liquid–liquid extraction as described in Section 2.3.

For the inhibition of 3 β HSD activity, trilostane (10 μ M) was added to the microsomal preparation and incubated on ice for 15 min prior to pre-incubation. For the inhibition of endogenous Cyt-b₅, the microsomal preparation was incubated on ice for 1 h prior to pre-incubation with purified anti-ovine Cyt-b₅ IgG (200 μ l), previously raised in our laboratory [10]. The activation of 3 β HSD activity by Cyt-b₅ was assessed by adding increasing amounts of purified Cyt-b₅ to obtain a range of ratios (endogenous Cyt-b₅:added Cyt-b₅) in the microsomal preparation. A 15 min incubation period was carried out on ice prior to pre-incubation.

2.8. Separation and quantification of steroids

High performance liquid chromatography was performed on a SpectraSYSTEM P4000 high performance liquid chromatograph (Thermo Separation™ products, San Jose, CA, USA) coupled to a SpectraSYSTEM AS3000 automatic injector (Thermo Separation™ products, San Jose, CA, USA) and a Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA). The substrates, PREG and DHEA, and their respective steroid metabolites were separated using a Phenomex® C₁₂ column at a flow rate of 1 ml min^{–1}. The ratio of scintillant to column element was 3:1. For the separation of PREG from PROG the mobile phase consisted of solvent A (methanol/water: 75%/25%) and solvent B (100% methanol). Steroids were eluted for 2 min with solvent A, followed by a linear gradient from 100% A to 100% B in 9 min and an isocratic elution

with solvent B for 2 min. DHEA and A4 were eluted with solvent A (methanol/water: 65%/35%) for 15 min, followed by a linear gradient from 100% A to 100% B (100% methanol) in 2 min and an isocratic elution with solvent B for 2 min. The injection volume of the samples was 90 μ l.

17-OHPREG and 17-OHPROG were separated by UPLC (ACQUITY UPLC, Waters, Milford, MA, USA) using a Waters UPLC BEH C₁₈ column (2.1 \times 50 mm, 1.7 μ m) at 50 °C and a flow rate of 0.4 ml min⁻¹ as previously described. An API Quattro Micro tandem mass spectrometer (Waters, Milford, MA, USA) was used for quantitative mass spectrometric detection as previously described [24].

2.9. Determination of kinetic parameters

Apparent K_m and V_{max} values for ovine 3 β HSD towards PREG, 17-OHPREG and DHEA were determined in COS-1 cells transiently cotransfected with either ovine 3 β HSD (0.5 μ g) and *holo*-b₅ (0.5 μ g) or ovine 3 β HSD (0.5 μ g) and the mammalian expression vector pCI-neo (0.5 μ g) (Promega, Madison, WI, USA), which contained no DNA insert. The metabolism of PREG, 17-OHPREG and DHEA was assayed, as described in Section 2.3, using six different substrate concentrations ranging from 0.5 to 8 μ M. Initial reaction rates (nmol/h/mg total protein) were determined by linear regression for each substrate and substrate concentration, using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California). A minimum of four time points were used for each rate determination with the *R*-squared value for all rate regressions being higher than 0.96. Michaelis–Menten graphs for each substrate were subsequently plotted in GraphPad Prism. Apparent K_m (μ M) and V_{max} values (nmol/h/mg total protein) were determined by performing Michaelis–Menten curve fits, using the non-linear regression function of the GraphPad Prism5 software. All statistical analyses were performed using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California) [17,18].

2.10. Kinetic modeling

A simplified generic bi-substrate rate equation for computational systems biology [25] was used to describe the kinetics of 3 β HSD during the conversion of Δ^5 steroids to their corresponding Δ^4 steroid products. The kinetic equations were cast in a system of ordinary differential equations (ODEs) that describe the rate of change of the Δ^5 steroid substrates and the Δ^4 steroid products. The system of ODEs was solved with the NDSolve function of Mathematica 6 (Wolfram Research Inc.). The constructed model was assigned arbitrary binding constant values. The initial concentration of the Δ^4 steroid product was set to 0, the NAD⁺/NADH ratio was set to a fixed constant and the initial Δ^5 steroid substrate concentration was varied over a given concentration range. The model was used to determine the initial rate of conversion of the Δ^5 steroid substrates to their corresponding Δ^4 steroid products at different initial Δ^5 steroid concentrations. Following the initial simulation, a second simulation was performed in which the K_{NAD^+} value was decreased by an arbitrary value. The results obtained from these simulations were tabulated and used to construct a double-reciprocal or Lineweaver–Burke plot.

3. Results

3.1. Effect of Cyt-b₅ on 3 β HSD activity in COS-1 cells

3.1.1. Caprine and ovine 3 β HSD activity towards PREG, 17-OHPREG and DHEA

Caprine and ovine 3 β HSD were both expressed in COS-1 cells and assayed for activity with 1 μ M PREG, 17-OHPREG and DHEA as substrates. Coexpression with *holo*-b₅ resulted in a significant

increase in the conversion of the three steroid substrates for both enzymes with a more pronounced difference in substrate conversion being observed at later time points (Fig. 1). In cells expressing caprine 3 β HSD the addition of *holo*-b₅ resulted in a significant increase of $\approx 15\%$ in the conversion of Δ^5 steroids to their corresponding Δ^4 products after 2 h (Figs. 1 and 2). In contrast, the increase in enzymatic activity of ovine 3 β HSD after 2 h in the presence of *holo*-b₅ did not follow the same trend for all substrates. PREG and DHEA metabolism were significantly increased by 13.5% (± 1.1 SEM) and 24% (± 0.4 SEM), respectively, while the effect on 17-OHPREG conversion was minimal (5% ± 1.4 SEM) (Figs. 1 and 2).

3.1.2. Ovine 3 β HSD activity towards PREG in the presence of 11 β HSD2 and trunc-b₅

In order to determine whether the observed stimulation of 3 β HSD activity by Cyt-b₅ was due to a non-specific membrane effect, ovine 3 β HSD was coexpressed with another microsomal enzyme (11 β HSD2) and assayed for activity towards PREG. The coexpression of ovine 3 β HSD with human 11 β HSD2 in COS-1 cells did not result in a significant change in activity when assayed with 1 μ M PREG. Conversely, the inclusion of *holo*-b₅ resulted in a significant ($p < 0.01$) increase in PREG metabolism (Fig. 3).

Further investigations into non-specific membrane effects necessitated the preparation of trunc-b₅ (1–89 AA), which would not associate with the ER membrane as it does not contain the membrane spanning domain [26,27]. Ovine 3 β HSD was subsequently cotransfected into COS-1 cells with trunc-b₅ and assayed for activity with 1 μ M PREG. Interestingly, cotransfection with trunc-b₅ significantly ($p < 0.001$) increased the activity of ovine 3 β HSD towards PREG (Fig. 3).

3.1.3. Effect of apo-b₅ on PREG metabolism

Further investigations into the augmentation of 3 β HSD activity by Cyt-b₅, were carried out by cotransfecting COS-1 cells with ratios ranging from 1:1 to 1:4 of ovine 3 β HSD to either *holo*-b₅ or *apo*-b₅ [28]. *Apo*-b₅, the H68A Cyt-b₅ mutant which cannot bind heme [19], was used to investigate the mechanism through which Cyt-b₅ augments 3 β HSD activity.

A significant ($p < 0.01$) increase in 3 β HSD activity towards PREG was observed with a concomitant increase in the ratio of 3 β HSD to both *holo*-b₅ and *apo*-b₅ to (Fig. 4). The 1:4 ratio resulted in maximal stimulation for both *holo*-b₅ and *apo*-b₅ with the greatest increase in stimulation of 3 β HSD activity being observed when the ratio was adjusted from 1:2 to 1:4. In addition, this increase in the stimulation of 3 β HSD activity was significantly greater ($p < 0.05$) in the case of *holo*-b₅ compared to that of *apo*-b₅ (Fig. 4).

3.1.4. Comparison of 3 β HSD and *holo*-b₅ expression levels in COS-1 cells using CFP and YFP fusion proteins

Differences in 3 β HSD concentration, which may arise from variations in transfection efficiency, could result in differences in 3 β HSD enzyme activity. The expression levels of 3 β HSD and *holo*-b₅ following cotransfection were therefore investigated using 3 β HSD-CFP and *holo*-b₅-YFP fusion proteins and the fluorescence of each protein in cells containing both fluorescent signals was subsequently analyzed. In addition, cells were cotransfected with 3 β HSD-CFP and *holo*-b₅-YFP in ratios varying from 1:1 to 1:4 to determine whether varying the amount of plasmid DNA used in the transfection of the cells could alter protein expression levels.

No significant difference was observed between the fluorescent signals for 3 β HSD-CFP or *holo*-b₅-YFP between the three independent cotransfection experiments (Fig. 5A). In addition, the ratio between *holo*-b₅-YFP and 3 β HSD-CFP fluorescence remained constant between the different experiments (Fig. 5B). Furthermore, a significant ($p < 0.001$) increase in the fluorescent signal for *holo*-b₅-YFP was observed with a concomitant increase in the ratio of

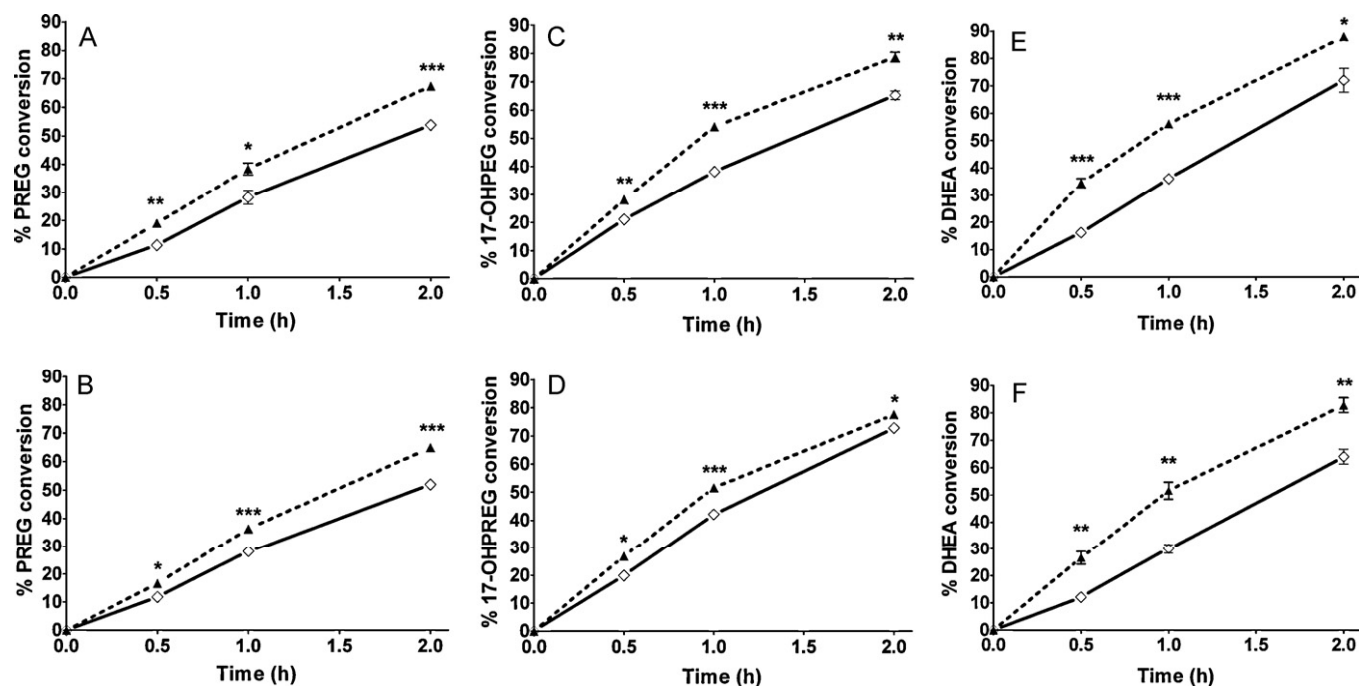


Fig. 1. Time courses of steroid metabolism by caprine and ovine 3 β HSD. The conversion of 1 μ M PREG, 1 μ M 17-OHPREG and 1 μ M DHEA by caprine (A, C and E) and ovine (B, D and F) 3 β HSD was compared in the presence of either *holo-b₅* (\blacktriangle) or pCneo (\diamond). Results were compared individually for each time point using an unpaired *t*-test. Results are expressed as the mean \pm SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *n* = 3).

3 β HSD-CFP to *holo-b₅*-YFP, while the fluorescent signal for 3 β HSD-CFP remained constant (Fig. 6).

3.1.5. Kinetic parameters of Angora and ovine 3 β HSD

Apparent K_m and V_{max} values of ovine 3 β HSD towards PREG, 17-OHPREG and DHEA were determined using non-linear regression in the presence and absence of *holo-b₅*. These data showed that the presence of *holo-b₅* had no influence on the apparent K_m values while significantly (*p* < 0.05) increasing the V_{max} values towards each of the respective substrates (Table 1).

3.1.6. The influence/effect of Cyt-*b₅* on co-factor binding

A simplified irreversible bi-substrate rate equation was used to construct a model in order to simulate the effect that alterations in the K_{NAD^+} value would have on the catalytic activity of 3 β HSD (i.e. V_{max}) [25,29]. Results from these simulations showed that by altering the K_{NAD^+} value the V_{max} value could be manipulated without altering the K_m value (Fig. 7). Decreasing the K_{NAD^+} value resulted in an increase in the V_{max} with no effect on the K_m , which corresponds to the experimental data generated in Section 3.1.5 (Fig. 7 and Table 1).

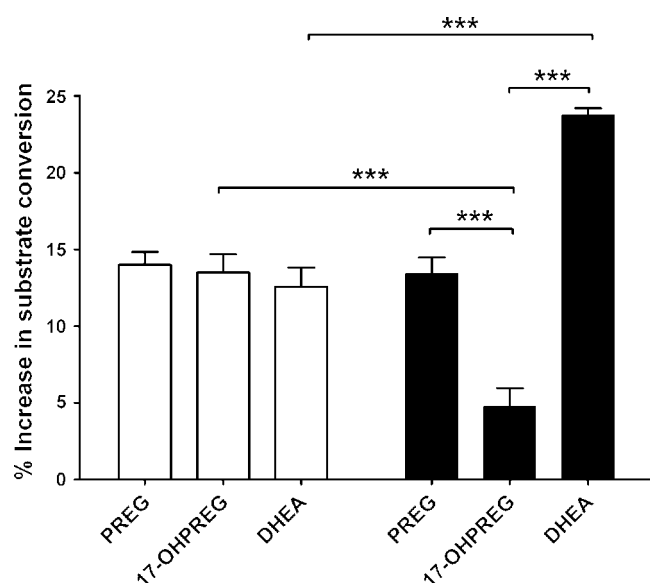


Fig. 2. Substrate conversion by 3 β HSD in the presence of *holo-b₅* in COS-1 cells. The percentage increase in substrate conversion by caprine (\square) and ovine (\blacksquare) 3 β HSD after 2 h was compared individually using an unpaired *t*-test. Results are expressed as the mean \pm SEM (****p* < 0.001, *n* = 3).

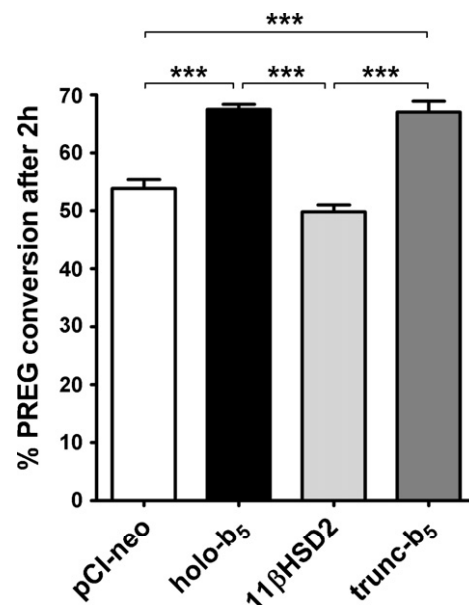


Fig. 3. Influence of *holo-b₅*, 11 β HSD2 and *trunc-b₅* on ovine 3 β HSD cotransfected in COS-1 cells. The conversion of PREG (1 μ M) was compared individually in the presence of constructs using an unpaired *t*-test. Results are expressed as the mean \pm SEM (****p* < 0.001, *n* = 3).

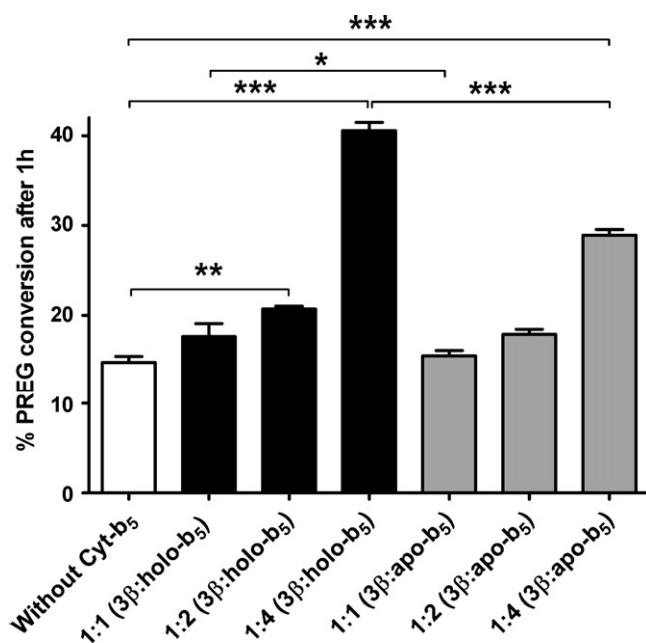


Fig. 4. Influence of *holo*- and *apo*-b₅ on substrate conversion in COS-1 cells. Cells were cotransfected with ovine 3βHSD and *holo*-b₅ or *apo*-b₅ in ratios ranging from 1:1 to 1:4. PREG (1 μM) conversion was compared individually with each ratio using an unpaired *t*-test. Results are expressed as the mean ± SEM, (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *n* = 3).

3.2. Effects of Cyt-b₅ on 3βHSD activity in ovine adrenal microsomes

3.2.1. Microsomal 3βHSD activity towards PREG, 17-OHPREG and DHEA

The influence of purified Cyt-b₅ on 3βHSD activity was further investigated in ovine adrenal microsomes. The P450 and Cyt-b₅ concentrations in the microsomal preparation, determined spectrophotometrically [22,23], were 0.5 nmol/mg protein and 0.06 nmol/mg protein respectively. These proteins were found to be expressed at a ratio of [P450]:[Cyt-b₅] ≈ 8:1 in the adrenal microsomes, which correlated with previous results obtained by Swart et al. [10] who showed the presence of low levels of endogenous Cyt-b₅ in ovine adrenal microsomes.

Ovine adrenal microsomes (0.35 μM P450) were assayed for 3βHSD activity towards PREG (10 μM), 17-OHPREG (20 μM) and DHEA (20 μM). The addition of purified Cyt-b₅ (0.4 μM), which

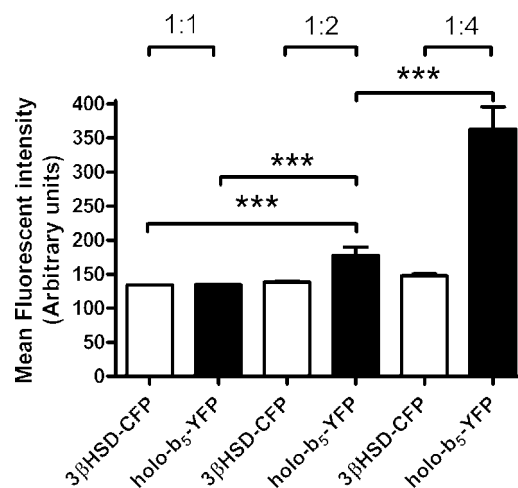


Fig. 6. Fluorescent intensity of *holo*-b₅-YFP and 3βHSD-CFP fusion proteins in COS-1 cells. Cells were cotransfected with 3βHSD-CFP and *holo*-b₅-YFP and in ratios ranging from 1:1 to 1:4. The fluorescent intensity of each fusion protein for each respective ratio was compared individually using an unpaired *t*-test. Results are expressed as the mean ± SEM (****p* < 0.001, *n* = 3).

represents a 10-fold increase in Cyt-b₅ concentration (relative to endogenous Cyt-b₅), resulted in a significant (*p* < 0.01) increase in PREG (10.3% ± 0.3 SEM) and DHEA (7.6% ± 0.2 SEM) conversion (Fig. 8A and C), while having no effect on 17-OHPREG conversion (Fig. 8B). The addition of trilostane (10 μM), a known 3βHSD specific inhibitor, abolished 3βHSD activity towards the three substrates (results not shown), confirming that the observed activity was specific for 3βHSD.

In addition to the incubation of the microsomal preparation with purified Cyt-b₅, the preparation was also incubated with anti-Cyt-b₅ IgG in order to determine the effect of endogenous Cyt-b₅ on microsomal 3βHSD activity. The microsomal preparation was incubated with purified anti-Cyt-b₅ IgG, previously raised in our laboratory [10], and assayed for activity with 10 μM PREG and 20 μM DHEA, as substrates. The addition of purified anti-Cyt-b₅ IgG significantly (*p* < 0.05) reduced 3βHSD activity towards PREG, while the addition of anti-HRP (horse radish peroxidase) IgG (control), had no effect on activity (Fig. 9A). A similar trend was observed for the metabolism of DHEA in the presence of purified anti-Cyt-b₅ IgG, however, the reduction in activity was not statistically significant (Fig. 9B).

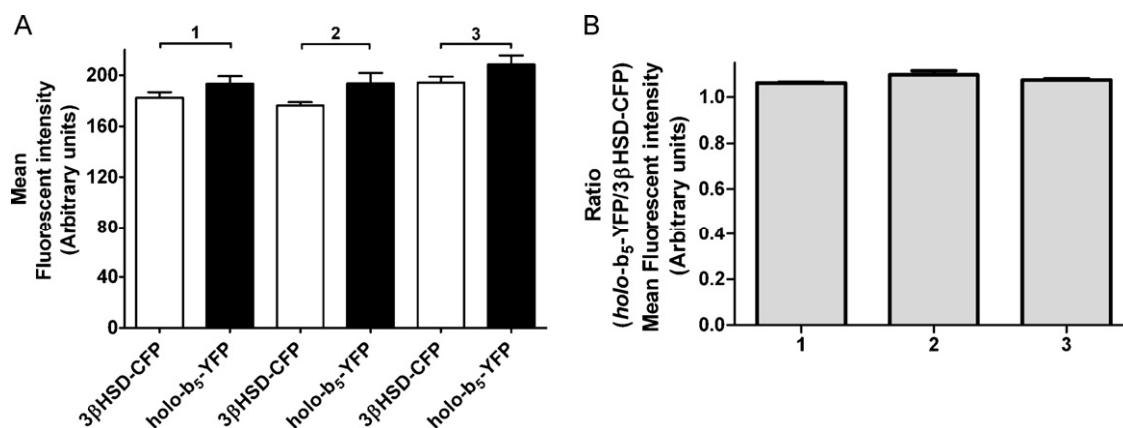


Fig. 5. The fluorescent intensity of 3βHSD-CFP and *holo*-b₅-YFP fusion proteins expressed in COS-1 cells. (A) The mean fluorescent intensity in three independent cotransfection experiments and (B) the ratio between the fluorescent intensity in three independent cotransfection experiments. The fluorescent intensities were compared using a one-way ANOVA followed by Bonferroni's multiple comparison post-test. Results are expressed as the mean ± SEM (*n* = 3).

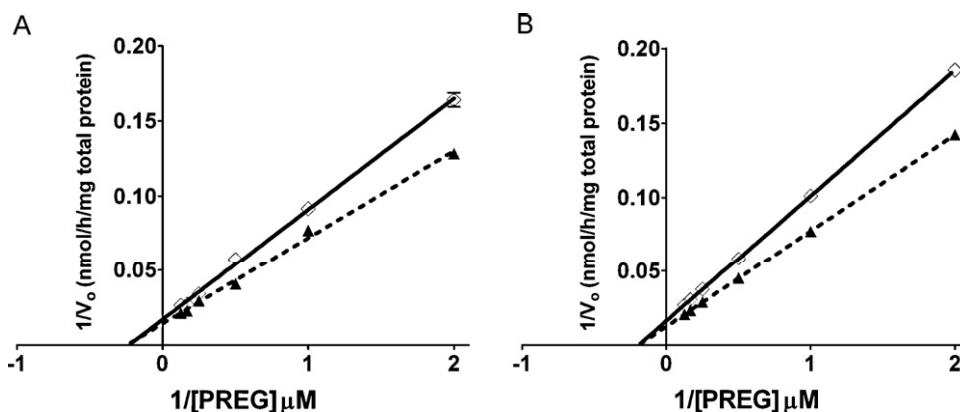


Fig. 7. Effect of Cyt-*b*₅ and varying K_{NAD^+} values on the kinetics of ovine 3βHSD in the conversion of PREG. (A) Comparison of ovine 3βHSD kinetics in the presence (▲) and absence (◇) of Cyt-*b*₅, determined experimentally. (B) Comparison of ovine 3βHSD kinetics, as predicted by the theoretical model, incorporating either high (◇) or low (▲) K_{NAD^+} values.

Table 1
Apparent K_m and the V_{max} values of ovine 3βHSD towards PREG, 17-OHPREG and DHEA in the presence and absence of *holo-b*₅. Apparent K_m (μM) and the V_{max} values (nmol/h/mg protein) were determined as described in Section 2.9 and are expressed as the mean ± SEM. The apparent K_m and V_{max} values of ovine 3βHSD are compared independently to that of ovine 3βHSD plus *holo-b*₅ for each substrate using an unpaired *t*-test.

Ovine 3βHSD	PREG		17-OHPREG		DHEA	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
– <i>holo-b</i> ₅	4.7 ± 0.5	63.3 ± 3.6	0.9 ± 0.2	101.6 ± 4.6	1.8 ± 0.2	22.4 ± 1.7
+ <i>holo-b</i> ₅	4.3 ^{ns} ± 0.4	73.6 [*] ± 3.8	1.2 ^{ns} ± 0.4	124.8 ^{***} ± 4.4	1.7 ^{ns} ± 0.3	172.1 ^{***} ± 8.2

^{ns} $p > 0.05$.

^{*} $p < 0.05$.

^{**} $p < 0.01$.

^{***} $p < 0.001$.

$n = 3$.

3.2.2. Effect of increased Cyt-*b*₅ concentrations on microsomal 3βHSD activity

3βHSD activity was assayed using 10 μM PREG in ovine adrenal microsomes (0.35 μM P450; 0.04 μM endogenous Cyt-*b*₅) which were incubated in the presence of 0.04–44 μM Cyt-*b*₅ added in ratios ranging from 1:1 to 1:100 of endogenous Cyt-*b*₅ to added

Cyt-*b*₅. A distinct increase in the conversion of PREG was observed in conjunction with an increase in the ratio of endogenous Cyt-*b*₅ to added Cyt-*b*₅. Maximal stimulation was observed at a ratio of 1:10. Increasing the ratio above 1:10 resulted in a significant ($p < 0.01$) decrease in the observed stimulation, with the 1:100 ratio showing no observable effect on PREG metabolism (Fig. 10).

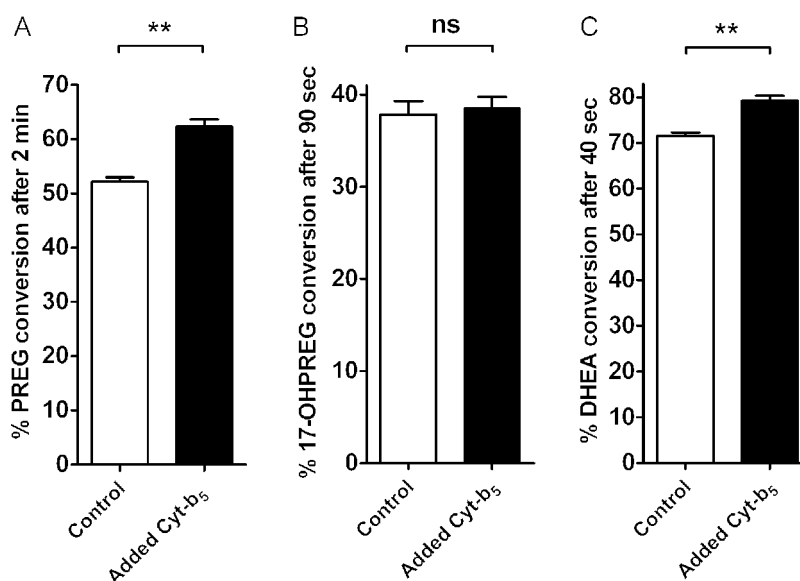


Fig. 8. Influence of added Cyt-*b*₅ on substrate conversion in ovine adrenal microsomes. (A) PREG, 10 μM; P450, 0.35 μM; endogenous Cyt-*b*₅, 0.04 μM; added Cyt-*b*₅, 0.4 μM; (B) 17-OHPREG, 20 μM; P450, 0.35 μM; endogenous Cyt-*b*₅, 0.04 μM; added Cyt-*b*₅, 0.4 μM and (C) DHEA, 20 μM; P450, 0.17 μM; endogenous Cyt-*b*₅, 0.02 μM; added Cyt-*b*₅, 0.2 μM. Substrate conversion was compared individually for each substrate in the presence/absence of purified Cyt-*b*₅ using an unpaired *t*-test. Results are expressed as the mean ± SEM (^{ns} $p > 0.05$, ^{**} $p < 0.01$, $n = 3$).

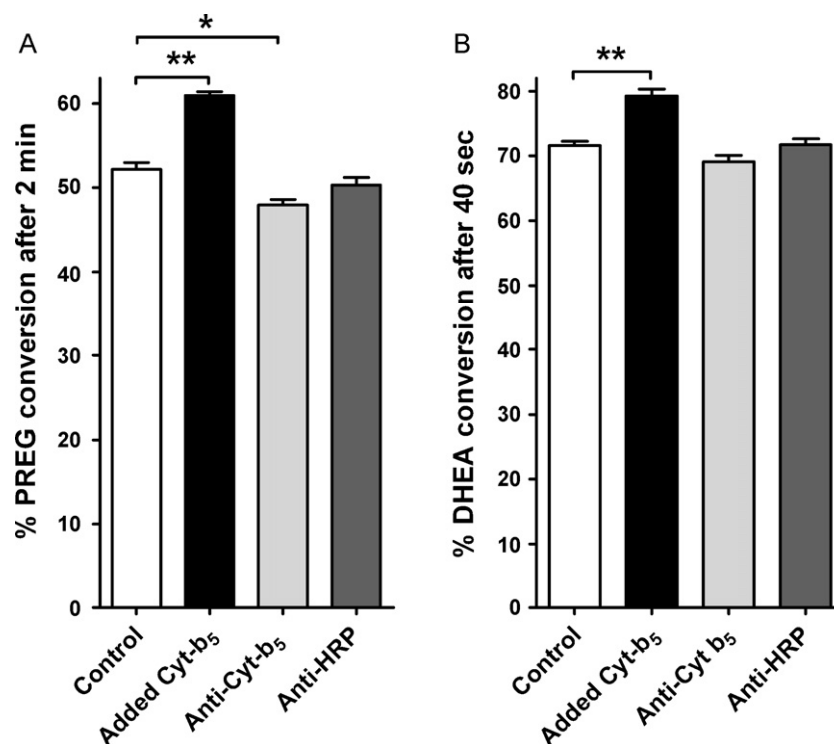


Fig. 9. Influence of anti-Cyt-b₅ IgG on substrate conversion in ovine adrenal microsomes. (A) PREG, 10 μ M; P450, 0.35 μ M; endogenous Cyt-b₅, 0.04 μ M; added Cyt-b₅, 0.4 μ M. (B). DHEA, 20 μ M; P450, 0.17 μ M; endogenous Cyt-b₅, 0.02 μ M; added Cyt-b₅, 0.2 μ M. Percentage conversion was compared individually for each substrate using an unpaired *t*-test. Results are expressed as the mean \pm SEM (**p* < 0.05, ***p* < 0.01, *n* = 3).

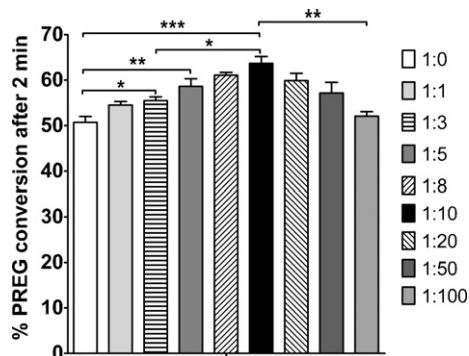


Fig. 10. PREG conversion in the presence of added Cyt-b₅ in ovine adrenal microsomes. PREG, 10 μ M; P450, 0.35 μ M; endogenous Cyt-b₅, 0.04 μ M; ratios of endogenous Cyt-b₅:added Cyt-b₅, 1:0–1:100. PREG conversion was compared individually using an unpaired *t*-test. Results are expressed as the mean \pm SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *n* = 3).

4. Discussion

In this study we showed Cyt-b₅, coexpressed with either caprine or ovine 3 β HSD in COS-1 cells, significantly increased the conversion of PREG, 17-OHPREG and DHEA to their respective Δ^4 products (Fig. 1). These data suggest that, in addition to its reported role in stimulating the lyase activity of CYP17A1, Cyt-b₅ may also augment 3 β HSD activity. The degree of stimulation was, however, different between the two species and between the respective steroid substrates (Fig. 2), indicating that Cyt-b₅ augmentation occurs in a species and substrate specific manner.

Augmentation of 3 β HSD activity by Cyt-b₅ was subsequently shown not to be attributed to non-specific membrane effects. The coexpression of 3 β HSD with 11 β HSD2 (localized to ER membrane) had no effect on 3 β HSD activity (Fig. 3) while trunc-b₅ (cytosolic) stimulated ovine 3 β HSD activity to the same extent

as *holo*-b₅ (Fig. 3). Coexpression of Cyt-b₅ with 3 β HSD therefore does not alter the ER membrane environment to such an extent that 3 β HSD becomes more accessible to steroid substrates and co-factors. Subsequent incubations of the microsomal preparations with anti-Cyt-b₅ IgG resulted in a significant reduction in 3 β HSD activity towards PREG (Fig. 9A), confirming Cyt-b₅'s augmentation of 3 β HSD. Although a similar trend was observed for DHEA metabolism, the reduction in activity by the addition of purified anti-Cyt-b₅ IgG was not statistically significant (Fig. 9B). This may be attributed to the conversion rate of DHEA with more than 70% A4 formation after 40 s even though the reaction conditions were optimized, implying that the reaction rate may no longer be linear, thus reducing the observable effect of the anti-Cyt-b₅ IgG.

The addition of purified Cyt-b₅ to microsomal preparations significantly increased 3 β HSD activity towards PREG with maximum stimulation at 1:10 (endogenous Cyt-b₅:added Cyt-b₅). However, as the ratio was adjusted beyond the optimum of 1:10 the degree of stimulation was significantly reduced (Fig. 10). This was previously observed in the stimulation of CYP17A1 activity by Cyt-b₅. Auchus et al. [30] and Soucy and Luu-The [28] reported that the stimulated lyase activity was substantially reduced in the presence of excess Cyt-b₅ and concluded that this was due to electron scavenging by Cyt-b₅ from POR at higher ratios of Cyt-b₅. However, since 3 β HSD does not require electrons from an external electron donor to perform its catalytic function, this seems an unlikely explanation for the reduced stimulation.

The addition of purified Cyt-b₅ had no significant effect on 17-OHPREG conversion (Fig. 8B). These results correlate with the results obtained in COS-1 cells where the greatest increase in substrate conversion by ovine 3 β HSD in the presence of Cyt-b₅ was observed for PREG and DHEA, with a comparatively small increase observed for 17-OHPREG conversion (Figs. 1 and 2). The apparent *V*_{max} value of ovine 3 β HSD towards 17-OHPREG in the absence of Cyt-b₅ is significantly greater than that of the enzyme towards PREG or DHEA (Table 1). The lack of apparent 3 β HSD stimulation

by Cyt- b_5 in the conversion of 17-OHPREG could be attributed to the activity of 3 β HSD towards 17-OHPREG already being high, and thus the degree to which the enzyme may be stimulated is reduced.

Traditionally, Cyt- b_5 is believed to augment the activity of specific cytochromes P450 via direct electron transfer. However, Auchus et al. [30] demonstrated that apo- b_5 was able to stimulate the 17,20 lyase activity of CYP17A1 and proposed an allosteric mechanism. Cyt- b_5 also plays an allosteric role in the catalysis of various other cytochrome P450 enzymes which include CYP3A4, CYP3A5 and CYP2A6 [31,32]. Guryev et al. [19], however, suggested that the stimulatory effect of apo- b_5 on CYP17A1 and CYP3A4 was due to the transfer of excess heme to apo- b_5 generating holo- b_5 , resulting in the stimulatory effects observed. Unlike the cytochromes P450, 3 β HSD does not require the input of electrons from an external electron donor and it is therefore unlikely that electron transfer plays a role. We demonstrated that apo- b_5 stimulated the activity of 3 β HSD (Fig. 4), albeit to a lesser degree than holo- b_5 , implying that Cyt- b_5 exerts an allosteric effect on 3 β HSD. The lower stimulatory effect of apo- b_5 is likely due to conformational changes in the protein resulting from the removal of the heme moiety [33,34].

The kinetic constants (K_m and V_{max}) of ovine 3 β HSD revealed that Cyt- b_5 had no influence on the apparent K_m values, but significantly ($p < 0.05$) increased the V_{max} values towards each of the Δ^5 substrates (Table 1). Unlike the K_m values, which are not dependent on enzyme concentration, the V_{max} values may vary as a result of differing transfection efficiencies. We previously demonstrated, using quantitative real-time PCR, that transient transfections of caprine or ovine 3 β HSD in COS-1 cells consistently yield comparable mRNA levels [18]. In addition, we now show that the level of fluorescence for both the 3 β HSD-CFP and holo- b_5 -YFP fusion proteins, as well as the ratio between the two, remained relatively constant following three independent experiments (Fig. 5). Similar levels of 3 β HSD and holo- b_5 are thus present, suggesting that it is unlikely that variations in the expression levels are responsible for the differences observed in 3 β HSD activity. Furthermore, this confirms that the cotransfection of 3 β HSD and holo- b_5 results in the expression of both enzymes in the same cell. In addition, the level of holo- b_5 -YFP fluorescence was found to increase significantly ($p < 0.001$) as the ratio of 3 β HSD-CFP:holo- b_5 -YFP plasmid DNA used for transfection was adjusted from 1:1 to 1:4, while the level of 3 β HSD-CFP fluorescence remained constant in these cells (Fig. 6). These results correlate with data presented in Fig. 4 where a relatively small increase in 3 β HSD activity was observed when the ratio of holo- b_5 :3 β HSD was adjusted to 1:2. However, a greater increase in 3 β HSD activity was observed with the increased ratio of 1:4. Similarly, in Fig. 6, a relatively small increase in the level of holo- b_5 -YFP fluorescence was observed when the ratio of 3 β HSD-CFP:holo- b_5 -YFP was adjusted to 1:2 while a significantly greater increase was observed at 1:4. These results suggest that an increase in plasmid DNA can increase the level of holo- b_5 expressed in COS-1 cells, resulting in an increase in the level of 3 β HSD stimulation, as observed in Fig. 4.

While the unchanged apparent K_m values for the steroid substrates indicate that significant conformational changes of the active site are unlikely, subtle changes brought about by the putative allosteric interactions between Cyt- b_5 and 3 β HSD, may impact on co-factor binding. It is possible that the binding of NAD $^+$ may, be affected resulting in the stimulation of the dehydrogenase activity without influencing substrate affinity. Using a simplified irreversible bi-substrate rate equation we demonstrated that decreasing the K_{NAD^+} value results in an increase in the V_{max} value without altering the K_m value, corresponding to the experimental data (Fig. 7). Increasing the rate of the dehydrogenase reaction would lead to an increase in the overall rate of the enzyme as the isomerase activity has been shown to be ≈ 10 -fold greater than the

dehydrogenase activity [35–37]. The data generated by these simulations, in conjunction with the experimental data, thus strongly suggests that an allosteric interaction between 3 β HSD and Cyt- b_5 results in an increase in the affinity of 3 β HSD for NAD $^+$, stimulating the dehydrogenase reaction, resulting in an increase in the overall catalytic activity.

The biosynthesis of A4, a vital precursor of sex steroids, requires the coexpression of Cyt- b_5 , 3 β HSD and CYP17A1 [38]. While significant levels of circulating A4 are believed to originate in the adrenal cortex [39–41] the expression of 3 β HSD is low in the zona reticularis (ZR), which specifically expresses Cyt- b_5 . Conversely, 3 β HSD is expressed in the zona fasciculata (ZF) and zona glomerulosa (ZG) in the absence of significant Cyt- b_5 expression [42,43]. Nakamura et al. [44] recently highlighted the importance of Cyt- b_5 and 3 β HSD coexpression in the production of A4 and identified a layer of adrenocortical cells located at the border between the ZF and ZR that coexpress both 3 β HSD and Cyt- b_5 in the presence of CYP17A1. Until now, Cyt- b_5 was only thought to play a role in the stimulation of the 17, 20-lyase reaction catalyzed by CYP17A1, however, based on the results presented in this paper we suggest that Cyt- b_5 may also stimulate 3 β HSD activity, thereby promoting the formation of A4 from DHEA.

In addition to the adrenal cells described above, 3 β HSD and Cyt- b_5 are coexpressed in other prominent steroidogenic organs such as the kidneys, testis (Leydig cells) and ovaries (theca cells) [2,43,45,46], suggesting that Cyt- b_5 may play a more extensive role in steroidogenic reactions than originally thought. The augmentation of 3 β HSD activity by Cyt- b_5 presented in this paper can, therefore not be ignored. Future studies will reveal the full physiological impact of such an augmentation.

5. Conclusions

The data presented in this study provides clear evidence that 3 β HSD activity is specifically augmented by Cyt- b_5 . This augmentation was further also shown to differ between closely related species and between substrates, suggesting augmentation occurs in a substrate and species specific manner. Furthermore, the data suggests that this augmentation is most likely allosteric in nature, resulting in a decreased K_{NAD^+} value leading to an overall increase in enzymatic activity.

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5.3 Conclusion

The results presented in this study clearly show that 3 β HSD activity is specifically augmented by Cyt-b₅ and represents the first documentation of such augmentation in any species. In addition, this augmentation was shown to differ between substrates and closely related species, suggesting augmentation occurs in a substrate and species specific manner. Furthermore, the data suggests that this augmentation is most likely allosteric in nature, resulting in a decreased K_{NAD^+} value. It was therefore decided to investigate the mechanism through which Cyt-b₅ augments 3 β HSD activity. This investigation is presented in the following chapter.

CHAPTER 6

ALLOSTERIC INTERACTION BETWEEN 3 β -HYDROXYSTEROID DEHYDROGENASE/ Δ^5 - Δ^4 ISOMERASE AND CYTOCHROME b₅ INFLUENCES CO-FACTOR BINDING

6.1 Introduction

The data presented in the previous chapter clearly indicated that Cyt-b₅ significantly influences 3 β HSD activity during steroidogenesis. We hypothesized that this augmentation is most likely allosteric in nature as *apo*-b₅ was shown to stimulate 3 β HSD activity. In addition, kinetic studies in COS-1 cells indicated that Cyt-b₅ had no influence on the apparent K_m values whilst significantly increasing the apparent V_{max} values of the enzyme for each of the Δ^5 substrates. These results could be replicated *in silico* using a simplified irreversible bi-substrate rate equation by decreasing the K_{NAD}^+ value, suggesting that augmentation via an allosteric mechanism might result in a decreased K_{NAD}^+ value.

The aim of this study was therefore to investigate the mechanism through which Cyt-b₅ augments 3 β HSD activity. To achieve this aim the influence of Cyt-b₅ on the individual dehydrogenase and isomerase activities of 3 β HSD as well as co-factor binding had to be determined using purified enzyme. Consequently, 3 β HSD was overexpressed in SF-9 cells using a baculovirus expression system and purified with a detergent based enzyme purification method. Both substrate and co-factor kinetics were independently determined for both the dehydrogenase and isomerase activities using the purified enzyme, in the presence and absence of purified ovine liver Cyt-b₅. In addition, 3 β HSD-eCFP and Cyt-b₅-eYFP fusion proteins were expressed in COS-1 cells and analyzed for FRET. The results of the study have been published in the FASEB journal and are presented in the article included in this chapter (Goosen et al., 2012). [Reprinted with permission from the FASEB journal]

Allosteric interaction between 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase and cytochrome b_5 influences cofactor binding

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ABSTRACT The biosynthesis of steroid hormones, essential to the survival of all mammals, is dependent on the activity of 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3β HSD). 3β HSD activity is, in turn, influenced by cytochrome- b_5 (Cyt- b_5). However, the mechanism through which this occurs is unknown. In this study, we investigated this mechanism by evaluating the influence of Cyt- b_5 on the dehydrogenase and isomerase activities of 3β HSD. *Capra hircus* 3β HSD was overexpressed in SF-9 cells, using a baculovirus expression system, and purified. Substrate and cofactor kinetics were determined spectrophotometrically in the presence and absence of purified *Ovis aries* liver Cyt- b_5 . Nonspecific enzyme activity was evaluated by zero-enzyme, -substrate, and -cofactor blanks. Fusion proteins, 3β HSD-eCFP, and Cyt- b_5 -eYFP were subsequently coexpressed in COS-1 cells and analyzed for FRET. A CFP-YFP fusion protein served as positive control, while coexpression of 3β HSD-eCFP and cytochrome P450 17 α -hydroxylase/17,20 lyase-eYFP (CYP17A1-eYFP) served as negative control. Results showed Cyt- b_5 to decrease the K_{m,NAD^+} value of 3β HSD ≈ 3.5 -fold while increasing the $V_{max,app}$ of the dehydrogenase reaction $\approx 17\%$. FRET analysis showed COS-1 cells coexpressing 3β HSD-eCFP and Cyt- b_5 -eYFP to exhibit a FRET signal ≈ 9 -fold greater than that of the negative control. These results indicate that Cyt- b_5 augments 3β HSD activity via an allosteric mechanism by increasing the affinity of the enzyme toward NAD^+ .—Goosen, P., Swart, A. C., Storbeck, K.-H., Swart, P. Allosteric interaction between 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase and cytochrome b_5 influences cofactor binding. *FASEB J.* 27, 000–000 (2013). www.fasebj.org

Key Words: adrenal steroidogenesis • dehydroepiandrosterone • androstenedione • UPLC-MS/MS • FRET • NAD^+

IN MAMMALS, STEROID HORMONES play a vital role in mediating a variety of physiological processes, including electrolyte and water balance (mineralocorticoids), carbohydrate metabolism (glucocorticoids), and sexual development (sex steroids). The mineralocorticoids and glucocorticoids are synthesized primarily in the adrenal cortex, while the sex steroids are principally produced in the gonads (1, 2). All steroid hormones are synthesized sequentially from cholesterol by a number of steroidogenic cytochrome P450s (CYPs) and hydroxysteroid dehydrogenases (HSDs), with 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3β HSD) playing a pivotal role in the synthesis of all classes of steroid hormones (3).

In the mammalian adrenal, 3β HSD catalyzes the conversion of the Δ^5 steroids, pregnenolone (PREG), 17-hydroxypregnenolone (17-OHPREG), and dehydroepiandrosterone (DHEA) to the corresponding Δ^4 steroids, progesterone (PROG), 17-hydroxyprogesterone (17-OHPROG), and androstenedione (A4) through sequential dehydrogenase and isomerase reactions (4, 5). In the first dehydrogenation reaction, NAD^+ is reduced to NADH with the formation of a Δ^{5-3} ketosteroid intermediate, which, together with NADH, remains bound to the enzyme. The bound NADH induces a conformational change in the enzyme, resulting in the subsequent isomerization of the bound intermediate, yielding the corresponding Δ^4 steroid product (Fig. 1 and refs. 5, 6).

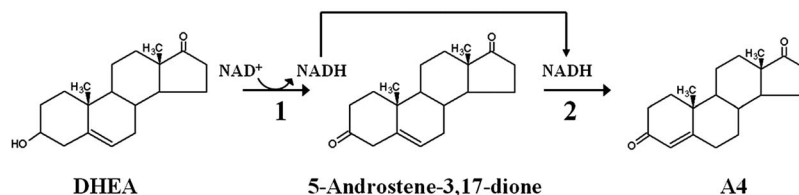
Substrate specificity and the catalytic activity of enzymes toward common substrates in steroid hormone biosynthesis are crucial in determining the flux of intermediates through the steroidogenic pathway (7–9). During adrenal steroidogenesis, 3β HSD and cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17A1) compete for the same steroid intermediates, as CYP17A1 catalyzes the hydroxylation of PREG and the subsequent lyase of the 17-OHPREG intermediate (1). It is known that cytochrome- b_5 (Cyt- b_5), a small electron transfer hemoprotein, influences this competition by selectively up-regulating the 17,20-lyase reaction while having a negligible effect on the 17 α -hydroxylase reac-

Abbreviations: 3β HSD, 3β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase; 17-OHPREG, 17-hydroxypregnenolone; 17-OHPROG, 17-hydroxyprogesterone; A4, androstenedione; BCA, bicinechonic acid; CYP, cytochrome P450; CYP17A1, 17 α -hydroxylase/17,20-lyase; Cyt- b_5 , cytochrome- b_5 ; DHEA, dehydroepiandrosterone; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; HSD, hydroxysteroid dehydrogenase; PREG, pregnenolone; PROG, progesterone

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Figure 1. Sequential dehydrogenase (1) and isomerase (2) reactions during the conversion of DHEA to A4 by 3 β HSD *via* a Δ^{5-3} keto intermediate, 5-androstene-3,17-dione.



tion (2, 10, 11). However, we recently made the novel discovery that, in conjunction to modulating CYP17A1 lyase activity, Cyt-b₅ also augments 3 β HSD activity (12), which represented the first documentation of Cyt-b₅ augmenting any member of the HSD enzyme family.

In our previous study, we showed that *apo*-b₅ (Cyt-b₅ void of heme) could stimulate 3 β HSD activity, suggesting a mechanism independent of direct electron transfer, a plausible deduction as 3 β HSD does not require electrons from an external electron donor to perform its catalytic function. Kinetic studies in COS-1 cells indicated that while Cyt-b₅ had no influence on the K_m values for each of the Δ^5 substrates, the apparent V_{max} ($V_{max,app}$) values were significantly increased. These results were replicated *in silico* using a simplified irreversible bisubstrate rate equation by decreasing the K_{m,NAD^+} value. Nonetheless, the manner in which Cyt-b₅ augments 3 β HSD is not fully understood. We therefore hypothesized that the augmentation of 3 β HSD activity, by Cyt-b₅, was most likely allosteric in nature, resulting in a decreased K_{m,NAD^+} value leading to an increase in the $V_{max,app}$ of the enzyme (12). This study was, however, carried out using ovine adrenal microsomes and recombinant 3 β HSD expressed in COS-1 cells and was only an indication of the overall effect of Cyt-b₅ on 3 β HSD.

The aim of this study was to investigate the influence of Cyt-b₅ on both the dehydrogenase and the isomerase activities of 3 β HSD as well as on cofactor binding using purified enzyme.

MATERIALS AND METHODS

Materials

MultiSite Gateway Pro cloning system, pcDNA3.2/V5/GW/D-TOPO plasmid vector, ER-Tracker, and Blue-White DPX dye were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Nucleobond AX plasmid purification kits were supplied by Macherey-Nagel (Duren, Germany). Wizard Plus SV Minipreps DNA purification kits, Wizard SV Gel and PCR Clean-Up System, and *NofI* and *XbaI* restriction enzymes were purchased from Promega Biotech (Madison, WI, USA). T4 DNA ligase, Rapid DNA pephosphorylation and ligation kit, *PstI* restriction enzyme, and NAD⁺ and NADH were supplied by Roche Applied Science (Mannheim, Germany). *Pfu* high-fidelity DNA polymerase was purchased from Fermentas Life Sciences (Burlington, ON, Canada). COS-1 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Mirus TransIT-LT1 transfection reagent was purchased

from Mirus Bio Corp. (Madison, WI, USA). *Spodoptera frugiperda* (SF-9) cells, linearized AcNPV Sapphire baculovirus DNA, pVL1392 transfer vector, and Sapphire insect transfection kit were supplied by Allele Biotechnology and Pharmaceuticals/Orbigen (San Diego, CA, USA). Fetal calf serum and bacterial culture medium were purchased from Highveld Biological (Lyndhurst, South Africa) and Difco Laboratories (Detroit, MI, USA), respectively. Grace's insect cell culture medium, yeastolate, penicillin-streptomycin, trypsin-EDTA, Dulbecco's PBS, and Pluronic F-68 were purchased from Gibco BRL (Gaithersburg, MD, USA). [7-³H]-PREG was purchased from PerkinElmer Life Sciences (Boston, MA, USA). 5-Androstene-3,17-dione was supplied by Steraloids (Newport, RI, USA). PREG, PROG, 17-OHPREG, 17-OHPROG, DHEA, A4, DMEM, lactalbumin hydrolysate, DTT, pepstatin A, apoprotinin, leupeptin, antipain, Igepal CO 720, and amphotericin B were supplied by Sigma Chemical Co. (St. Louis, MO, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest quality and obtained from reputable scientific supply houses. All methods were carried out according to the manufacturers' instructions, unless otherwise stated.

Preparation of recombinant baculovirus

Recombinant baculovirus was prepared using a recombinant transfer vector, pVL1392, containing 3 β HSD. The transfer vector was constructed using caprine 3 β HSD (GenBank accession no. EF524065), previously cloned from a caprine adrenal cortex homogenate (13). Briefly, the 3 β HSD cDNA sequence, representing the only known form of caprine 3 β HSD, was amplified using the primers shown in **Table 1**. The resulting fragment was gel purified using a Wizard SV Gel and PCR Clean-Up System and subcloned into the pVL1392 transfer vector, by digestion of complementary *NofI* and *XbaI* restriction sites, using the Rapid DNA dephosphorylation and ligation kit. Recombinant plasmid constructs were screened by restriction digest analysis using *PstI*, and appropriate clones were subsequently subjected to direct DNA sequence analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Johannesburg, South Africa).

SF-9 cells were grown as monolayers at 27°C in Grace's medium supplemented with 10% fetal calf serum, 3.3 mg/ml yeastolate, 3.3 mg/ml lactalbumin hydrolysate, 10 μ g/ml penicillin-streptomycin, and 0.25 μ g/ml amphotericin B (complete Grace's medium; ref. 14). Cells (1×10^6 cells/2 ml) were plated into a 35-mm culture dish and allowed to adhere for 30 min prior to transfection. Recombinant virus was obtained by cotransfecting the cells with linearized Sapphire baculovirus DNA (500 ng) and recombinant transfer vector pVL1392 containing 3 β HSD (1 μ g), using the Sapphire baculovirus DNA and transfection kit. At 5 d post-transfection, cell culture medium containing recombinant baculovirus was harvested by centrifugation at 1000 g (4°C) for 10 min. The supernatant containing the recombinant virus was subsequently subjected to 2 rounds of amplification, and the approximate titer value was determined using an endpoint

TABLE 1. *Oligonucleotide primers used for the construction of plasmid constructs*

Primer	Oligonucleotide sequence
3βHSD-eCFP/eYFP constructs	
3 β HSD-attB1 (sense)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCGGGCTGGAGCTGCCTG-3'
3 β HSD-attB5r (antisense)	5'-GGGGACAACCTTTTGTATACAAAGTTGTATGAATTTTGGTTTTCAGGGT-3'
eCFP/eYFP-attB5 (sense)	5'-GGGGACAACCTTTTGTATACAAAGTTGCGATGGTGAGCAAGGGCGAGG-3'
eCFP/eYFP-attB2r (antisense)	5'-GGGGACCACCTTTTGTACAAGAAAGCTGGGTATCCGGACTTGTACAGCTC-3'
Cyt-b₅-eCFP/eYFP constructs	
Cyt-b ₅ -attB5 (sense)	5'-GGGGACAACCTTTTGTATACAAAGTTGCGTCCGTGAGTTAAGAAATGGCC-3'
Cyt-b ₅ -attB2r (antisense)	5'-GGGGACCACCTTTTGTACAAGAAAGCTGGGTACTCCCTGGACCAAAGCAG-3'
eCFP/eYFP-attB1 (sense)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGATCCGCTAGCGCTAC-3'
eCFP/eYFP-attB5r (antisense)	5'-GGGGACAACCTTTTGTATACAAAGTTGTTCCGGACTTGTACAGCTC-3'
CYP17A1-eCFP/eYFP constructs	
CYP17A1-attB1 (sense)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGTGGGTGCTCTTGGGTGTC-3'
CYP17A1-attB5r (sense)	5'-GGGGACAACCTTTTGTATACAAAGTTGTTGAGGTGCTACCCTCAGCCTG-3'
eCFP/eYFP-attB5 (sense)	5'-GGGGACAACCTTTTGTATACAAAGTTGCGATGGTGAGCAAGGGCGAGG-3'
eCFP/eYFP-attB2r (antisense)	5'-GGGGACCACCTTTTGTACAAGAAAGCTGGGTATCCGGACTTGTACAGCTC-3'
Caprine 3βHSD-pVL1392	
LP (sense)	5'-AGAATTCAAAAAGCAGGCTCCGC-3'
RP (antisense)	5'-AGAATTTCGCGGGCCCTCTAGATC-3'

dilution assay. The resulting high-titer viral stock was protected from light and stored at 4°C.

Expression of recombinant 3 β HSD in Sf-9 cells

Two 1-L Sf-9 suspension cultures were grown at 27°C in a shaking incubator at 80 rpm in 2-L baffled Erlenmeyer flasks. The cells were grown to a density of 1.5×10^6 cells/ml in Grace's complete medium containing 0.1% Pluronic F-68 (14, 15). Infections were carried out with cells in log phase showing $\geq 97\%$ viability, as determined by the trypan blue dye exclusion assay using the Countess Automated Cell Counter (Invitrogen Life Technologies). Each culture was infected with recombinant virus at a multiplicity of infection ≥ 10 and incubated at 27°C. At 3 d postinfection, cells were harvested by centrifugation at 1000 *g* (4°C) for 10 min (14, 16). The cells were washed twice by resuspending the pellet in 100 ml Grace's medium, followed by centrifugation at 1000 *g* (4°C) for 10 min. Following the final centrifugation, the cells were resuspended in 100 ml homogenization buffer (0.1 M potassium phosphate, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.6 μ g/ml pepstatin A, 6.5 μ g/ml apoprotinin, 6.5 μ g/ml leupeptin, and 6.5 μ g/ml antipain). Cells were disrupted by sonication on ice in four 20-s bursts with 30-s intervals, using a Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Farmingdale, NY, USA; refs. 14, 17). The lysate was subsequently stored at -80°C until purification.

3 β HSD purification

3 β HSD was purified from the Sf-9 cell lysate, as described previously (15, 18, 19). All purification steps were performed at 4°C. Briefly, the Sf-9 cell lysate was thawed on ice, followed by centrifugation at 100,000 *g* (4°C) for 90 min. Following centrifugation, the pellet was resuspended in homogenization buffer using a small glass homogenizer, and the volume was adjusted to yield a 10 mg/ml protein preparation. 3 β HSD was solubilized by the addition of 6% sodium cholate to a final concentration of 0.6%, followed by gentle stirring at 4°C for 30 min. The suspension was subsequently centrifuged at 120,000 *g* (4°C) for 1 h. The resulting solubilized preparation was applied to a Fractogel TSK CM-650(s) column (Merck, Darmstadt, Germany) equilibrated with 0.02 M potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM

EDTA, 0.1 mM DTT, 0.01 mM NAD⁺, and 0.2% sodium cholate. The column was subsequently washed with 2 column volumes of equilibration buffer. 3 β HSD was eluted using 0.02 M potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01 mM NAD⁺, and 0.4% Igepal CO 720, and collected in 0.5-ml fractions.

3 β HSD enzyme activity was monitored during purification using a modified version of the microsomal 3 β HSD assay described previously (12). Briefly, incubations were performed at room temperature with the reaction mixture containing 30 μ l eluent and 0.5 mM NAD⁺ in 0.02 M potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 10 μ M PREG and [³H]PREG, and aliquots (50 μ l) were removed from the reaction mixture at specific time intervals. The steroid metabolites were subsequently extracted by liquid-liquid extraction using a 10:1 volume of dichloromethane to incubation medium. The samples were vortexed for 15 min and centrifuged at 500 *g* for 5 min. The water phase was aspirated off, and the dichloromethane phase was transferred to a clean test tube and dried under N₂. The dried steroid residue was redissolved in 120 μ l methanol prior to analysis. PREG and PROG were separated on a SpectraSystem P4000 high-performance liquid chromatograph (Thermo Separation, San Jose, CA, USA) coupled to a SpectraSystem AS3000 automatic injector (Thermo Separation) using a Phenomex C₁₂ column at a flow rate of 1 ml/min, as described previously (12). A Flo-One liquid scintillation spectrophotometer (Radiomatic, Tampa, FL, USA) was used for quantitative detection with the ratio of scintillant to column element set to 3:1.

The protein concentration of each fraction was determined using a Pierce BCA protein assay kit. The fractions exhibiting the highest specific 3 β HSD activity (data not shown) were pooled and subjected to a second round of column purification. The fractions with the highest specific 3 β HSD activity were subsequently pooled and stored at -80°C. Following SDS-PAGE analysis, the protein band corresponding to a 42-kDa apparent mass was excised from the gel and subjected to analysis by mass spectrometry.

Mass spectrometry of purified protein

Gel slices containing the protein were rinsed twice with water and incubated in 50% acetonitrile for 10 min. The acetonitrile

trile was replaced with 50 mM ammonium bicarbonate and incubated for 10 min. This step was repeated twice, and the slices were subsequently incubated in 100% acetonitrile until they turned white. The gel was dried *in vacuo*, after which proteins were reduced in 10 mM DTT for 1 h at 57°C. This was followed by brief washing with ammonium bicarbonate, followed by 50% acetonitrile, before the protein was alkylated with 55 mM iodoacetamide for 1 h in the dark. Following alkylation, the gel was washed with 50 mM ammonium bicarbonate for 10 min, followed by 50% acetonitrile for 20 min, before being dried *in vacuo*. The proteins were digested overnight with 20 μ l trypsin solution (10 ng/ μ l) at 37°C. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% formic acid for 30 min, dried, and stored at -20°C. The peptides were redissolved in 5% acetonitrile in 0.1% formic acid (10 μ l) prior to nano-LC-MS/MS.

Peptide samples were analyzed on a Thermo Scientific Easy-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanoelectrospray source. Separation was performed on an Easy-Column precolumn (2 cm, i.d. 100 μ m, 5 μ m, C18) followed by an Easy-Column column (10 cm, ID 75 μ m, 3 μ m, C18) with a flow rate of 300 nl/min. The mobile phase consisted of solvent A (100% water; 0.1% formic acid) and solvent B (100% acetonitrile; 0.1% formic acid). The peptides were eluted using a gradient from 5 to 40% B in 20 min, followed by a gradient from 40 to 80% B in 5 min, and kept at 80% B for 10 min. The mass spectrometer was operated in data-dependent mode, switching automatically between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package (Thermo Scientific). The precursor ion scan MS spectra (m/z 400-2000) were acquired in the Orbitrap with resolution $r = 60,000$, with the number of accumulated ions being 1×10^6 . The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 3×10^4) using collision-induced dissociation. The lock mass option (polydimethylcyclsiloxane; m/z 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 30-s exclusion duration. Mass spectrometry conditions were 1.5 kV, capillary temperature of 200°C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS, and an activation Q value of 0.25 and activation time of 10 ms were also applied for MS/MS.

The Thermo Proteome Discoverer 1.2.0.208 software package (Thermo Scientific) was used to identify proteins *via* automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra. Carbamidomethyl cysteine was set as fixed modification and oxidized methionine as variable modification. The precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.8 Da, while missed tryptic cleavages was limited to 2. Positive identification of proteins was limited to a minimum of 2 tryptic peptides/protein, which exhibited high to medium confidence as determined by Proteome Discoverer and a Mascot score greater than $P < 0.05$.

Spectrophotometric enzyme assays of purified 3 β HSD

The dehydrogenase and isomerase activities of purified 3 β HSD were assayed spectrophotometrically as described previously (6, 18). Cyt-b₅ was purified from ovine microsomal preparation as described previously (12). The activation of 3 β HSD activity by Cyt-b₅ was assessed over a range of ratios (Cyt-b₅:3 β HSD) in incubations containing DHEA (100 μ M), NAD⁺ (30 μ M), 3 β HSD (0.01 mg), and Cyt-b₅ (0-0.04 mg) in 0.02 M potassium phosphate buffer (pH 7.4) at 27°C.

Kinetic parameters for the dehydrogenase activity of puri-

fied 3 β HSD toward DHEA were determined in incubations containing DHEA (1-100 μ M), NAD⁺ (0.1 mM), and 3 β HSD (0.01 mg) in 0.02 M potassium phosphate buffer (pH 7.4) at 27°C. Kinetic parameters for the dehydrogenase cofactor (NAD⁺) were determined in incubations containing NAD⁺ (10-100 μ M), DHEA (100 μ M), and 3 β HSD (0.01 mg) in 0.02 M potassium phosphate buffer (pH 7.4) at 27°C. The slope of the initial linear increase in absorbance at 340 nm over time (due to NADH formation) was used to determine dehydrogenase activity.

Kinetic parameters for the isomerase activity of purified 3 β HSD toward 5-androstene-3,17-dione were determined in incubations containing 5-androstene-3,17-dione (10-100 μ M), NADH (0.05 mM), and 3 β HSD (0.01 mg) in 0.02 M potassium phosphate buffer (pH 7.4) at 27°C. Kinetic parameters for the isomerase cofactor (NADH) were determined in incubations containing NADH (2-50 μ M), 5-androstene-3,17-dione (100 μ M) and 3 β HSD (0.01 mg) in 0.02 M potassium phosphate buffer (pH 7.4) at 27°C. Isomerase activity was measured by the initial linear increase in absorbance at 241 nm (due to A4 formation) over time. Changes in absorbance at 340 nm were measured with a PowerWave 340 microplate spectrophotometer (BioTek Instruments, Winooksi, VT, USA), while changes in absorbance at 241 nm were measured with a Varioskan multiplate spectrophotometer (Thermo Scientific, Bremen, Germany). In all spectrophotometric assays, nonspecific enzyme activity was evaluated in reaction mixtures without substrate and without enzyme, as described previously (6, 18). Kinetic parameters (K_m , V_{max}) were determined by nonlinear regression using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). K_{cat} values (min^{-1}) were calculated from the V_{max} values ($\text{nmol}/\text{min}/\text{mg}$) and represent the maximal substrate turnover rate ($\text{nmol}/\text{min}/\text{nmol}$ enzyme). All kinetic parameters were determined in the absence and presence of Cyt-b₅ (0.01 mg) at a ratio of 1:1 (Cyt-b₅:3 β HSD).

3 β HSD activity assay in transiently transfected COS-1 cells

Chimeric constructs 3 β HSD-eCFP, 3 β HSD-eYFP, CYP17A1-eCFP, CYP17A1-eYFP, and Cyt-b₅-eYFP were prepared using the MultiSite Gateway Pro cloning system. Caprine 3 β HSD (GenBank accession no. EF524065), caprine CYP17A1 (GenBank EF524063), and caprine Cyt-b₅ (GenBank EF524066) previously cloned into the pcDNA3.2/V5/GW/D-TOPO vector served as a template (13, 20). eCFP-GR and eYFP-GR vector constructs were used as templates for the amplification of eCFP and eYFP, respectively. All the primers used in the amplification reactions are shown in Table 1. All constructs were subjected to direct DNA sequence analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

COS-1 cells were grown at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 0.12% NaHCO₃. Competent cells (1.0×10^5 cells/1 ml/well) were plated into 12-well plates 24 h prior to transfection. The cells were transiently cotransfected with plasmid constructs (1 μ g) using TransIT-LT1 transfection reagent: 3 β HSD (0.5 μ g) and pCI-neo (0.5 μ g), without DNA insert; 3 β HSD (0.5 μ g) and Cyt-b₅ (0.5 μ g); 3 β HSD-eCFP (0.5 μ g) and Cyt-b₅ (0.5 μ g); 3 β HSD (0.5 μ g) and Cyt-b₅-eYFP (0.5 μ g) as well as 3 β HSD-eCFP (0.5 μ g), and Cyt-b₅-eYFP (0.5 μ g).

Enzyme activity was determined by assaying DHEA (1 μ M) metabolism 72 h post-transfection. Aliquots (500 μ l) were removed at specific time intervals from the assay mixtures, and steroid metabolites were subsequently extracted as described in above and analyzed by UPLC-MS/MS. On completion of each experiment, the cells were washed and collected in phosphate buffer (0.1 M, pH 7.4). The cells were homog-

enized with a small glass homogenizer, and the total protein content of the homogenate was determined using the Pierce BCA protein assay kit.

DHEA and A4 were separated by UPLC (Acquity UPLC; Waters, Milford, MA, USA) using a Waters UPLC BEH C₁₈ column (2.1×50 mm, 1.7 µm) at 50°C and a flow rate of 0.4 ml/min, as described previously (21). A Xevo triple quadrupole mass spectrometer (Waters) was used for quantitative mass spectrometric detection. All steroids were analyzed in multiple reaction monitoring mode using an electrospray in the positive ionization mode (ESI⁺). The following settings were used: capillary voltage of 2.8 kV, cone voltage 15–35 V, collision energy 4–32 eV, source temperature 100°C, desolvation temperature 500°C, desolvation gas 1000 L/h, cone gas 50 L/h, and dwell time 0.002 s. Calibration curves were constructed by using weighted (1/*x*²) linear least squares regression. Data were collected with the MassLynx 4.0 software program.

Colocalization of 3βHSD-eCFP and Cyt-b₅-eYFP fusion proteins in COS-1 cells

Competent COS-1 cells (1.5×10³ cells/250 µl/well) were plated into an 8-well chambered coverglass plate 24 h prior to transfection. The cells were transiently cotransfected with chimeric plasmid constructs 3βHSD-eCFP and Cyt-b₅-eYFP (0.15 µg) using TransIT-LT1 transfection reagent. At 48 h post-transfection, the cells were analyzed in a temperature-controlled chamber (37°C) of an Olympus CellR system attached to an IX 81 inverted fluorescence microscope (Olympus Corp., Tokyo, Japan) equipped with an F-view-II cooled CCD camera (Soft Imaging System; Olympus). The light source was a 150-W xenon lamp, part of an MT20 excitation system. Cells were observed with an ×60 oil-immersion objective, and the CellR imaging software was used for image acquisition and analysis. The endoplasmic reticulum (ER) was visualized using ER-Tracker Blue-White DPX dye. A 360-nm excitation filter was used to acquire the ER tracker images, and emission was collected using a UBG triple-bandpass emission filter cube (Chroma Technology Corp., Bellows Falls, VT, USA). The YFP filter set excited at S500/20x, and emission was detected at S535/30m, while the CFP filter set excited at S430/25x, and emission was detected at S470/30m. Z-stack image frame acquisition was performed using an Olympus Plan Apo N ×60/1.4 oil objective and the CellR imaging software, with a step width of 0.5 µm. Images were processed, background was subtracted, and signal through the UBG channel was colocalized with either the CFP or the YFP signal, using the CellR software.

Fluorescence resonance energy transfer (FRET) analysis of fusion proteins in COS-1 cells

Competent COS-1 cells (2.0×10⁵ cells/2 ml/well) were plated into 6-well plates, with each well containing a small glass coverslip. After 24 h, the cells were transiently cotransfected using TransIT-LT1 transfection reagent with the following vector constructs: 3βHSD-eCFP (1 µg) and CYP17A1-eYFP (1 µg) (–V_e); CFP-YFP (1 µg) and pCI-neo (1 µg) (+V_e); 3βHSD-eCFP (1 µg); and 3βHSD-eYFP (1 µg); as well as 3βHSD-eCFP (1 µg) and Cyt-b₅-eYFP (1 µg). At 48 h post-transfection FRET was analyzed on an Olympus CellR system attached to an IX 81 inverted fluorescence microscope (Olympus). The YFP filter set excited at S500/20x (Chroma Technology), and emission was detected at S535/30m, whereas the CFP filter set excited at S430/25x, and emission was detected at S470/30m. CFP, YFP, and FRET images were acquired, and FRET was detected using a filter set with

S430/25x excitation and S535/30m emission. The signals measured in the FRET channel were corrected for crosstalk from the cyan and yellow channel using the equation $n\text{FRET} = \text{FRET signal} - (a \times \text{YFP signal}) - (b \times \text{CFP signal})$, where *n* is normalized FRET, and *a* and *b* were determined by measuring the crossover into the FRET channel of the YFP and CFP signals, respectively, in cells expressing each fusion protein on its own (22). Background subtraction was carried out using an area where no cells were present.

RESULTS

Influence of Cyt-b₅ on purified 3βHSD

Expression and purification of caprine 3βHSD

Kinetic parameters describing, first, the dehydrogenase reaction, followed by the isomerase reaction, had to be determined in order to fully characterize the influence of Cyt-b₅ on 3βHSD activity. Such analysis required purified enzyme that could be quantified and used to determine not only the conversion of the initial substrate but also the intermediate. Since COS-1 cells are unsuitable for these kinetic assays, caprine 3βHSD was overexpressed using a baculovirus expression system and subsequently subjected to a detergent-based purification method (6, 14, 15, 18, 19). **Figure 2** shows that a protein ≈42 kDa in size was successfully expressed in Sf-9 cells and purified from the cell lysate. The identity of the purified protein was subsequently confirmed as 3βHSD by mass spectrometry (**Table 2**). In addition, 3βHSD activity toward PREG (1 µM) was confirmed in the cell lysate of infected cells, as well as in the purified sample (**Fig. 3**).

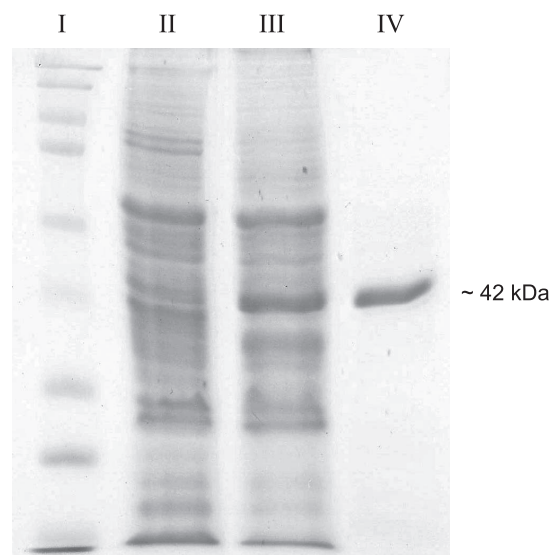


Figure 2. SDS-PAGE analyses of 3βHSD expressed in Sf-9 cells. Lanes were loaded with 30 µg protein, and the bands were visualized with Coomassie blue staining. Lane I, marker; lane II, cell lysate of infected Sf-9 cells; lane III, solubilized fraction of Sf-9 cell lysate; lane IV, purified 3βHSD (~42.5 kDa).

TABLE 2. Peptide analysis of purified Angora β HSD

Parameter	Value
σ coverage	86.06
σ proteins (<i>n</i>)	3
σ unique peptides (<i>n</i>)	25
σ peptides (<i>n</i>)	26
σ PSMs (<i>n</i>)	393
A4 score	10,526.5
A4 coverage	86.06
A4 peptides (<i>n</i>)	26
A4 PSMs (<i>n</i>)	393
Amino acids (<i>n</i>)	373
MW (kDa)	42.5
Calculated pI	7.84

PSM, peptide-spectrum match.

Kinetic analysis of the dehydrogenase and isomerase activities of β HSD

The effect of Cyt- b_5 on the activity of β HSD was assessed spectrophotometrically in incubations containing DHEA (100 μ M), NAD⁺ (30 μ M), purified β HSD (0.01 mg), and purified Cyt- b_5 in ratios ranging from 1:1 to 4:1 (Cyt- b_5 : β HSD). A significant ($P < 0.01$) increase in β HSD activity was observed when Cyt- b_5 was added to the reaction mixture at a ratio of 1:1. Increasing the ratio above 1:1 resulted in smaller, but significant ($P < 0.05$), stimulation, up to a ratio of 4:1, at which Cyt- b_5 no longer stimulated β HSD activity (Fig. 4).

The influence of Cyt- b_5 on β HSD activity was further investigated by determining kinetic parameters for the dehydrogenase and isomerase reactions, using purified enzyme. The kinetic parameters measured for the dehydrogenase activity of β HSD for the substrate DHEA and the cofactor (NAD⁺) in the presence and

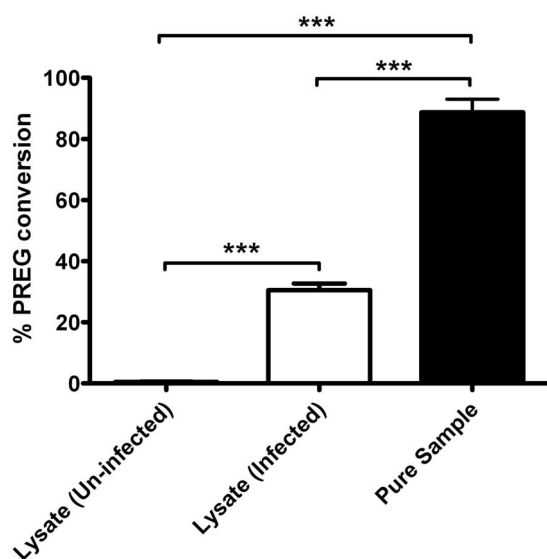


Figure 3. PREG metabolism by purified recombinant β HSD after 3 min. β HSD activity in infected and uninfected Sf-9 cell lysates and in pooled column fraction were compared individually using an unpaired *t* test. Results are expressed as means \pm SE (*n*=3). *** $P < 0.001$.

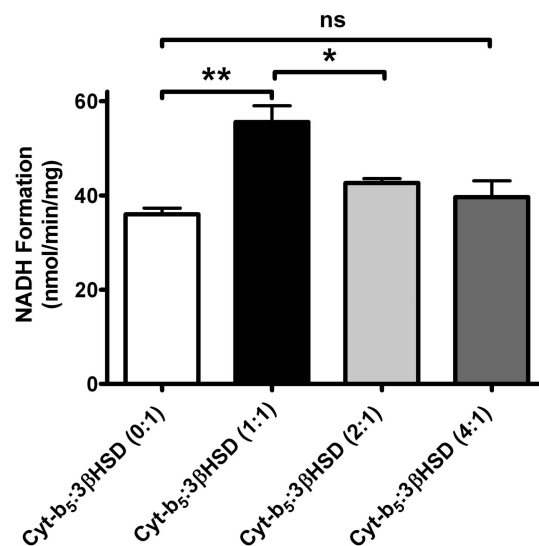


Figure 4. Rate of NADH formation in the presence of purified Cyt- b_5 . The dehydrogenase activity of purified β HSD was compared for each reaction individually over a range of Cyt- b_5 : β HSD ratios (0:1 to 4:1), using an unpaired *t* test. Results are expressed as means \pm SE (*n*=3). ns, not significant ($P > 0.05$). ** $P < 0.01$, *** $P < 0.001$.

absence of purified Cyt- b_5 (1:1 ratio), are summarized in Table 3. The kinetic parameters determined for DHEA are representative of apparent values under the assay conditions, as the NAD⁺ was not truly saturating. No significant difference was observed between the apparent $K_{m,DHEA}$ ($K_{m,app,DHEA}$) values of β HSD in the presence or absence of Cyt- b_5 , while a significant ($P < 0.05$) increase in $V_{max,app}$ was observed in the presence of Cyt- b_5 (Fig. 5A). Furthermore, significant increases in the apparent K_{cat} ($K_{cat,app}$; $P < 0.05$) and substrate utilization efficiency ($K_{cat,app}/K_{m,app,DHEA}$; $P < 0.05$) were observed in the presence of Cyt- b_5 . In contrast to the $K_{m,app,DHEA}$, the presence of Cyt- b_5 resulted in a ≈ 3.5 -fold decrease in the K_{m,NAD^+} , while having no observable effect on the V_{max} or K_{cat} values (Table 3 and Fig. 5B). The presence of Cyt- b_5 , did however, significantly ($P < 0.001$) increase the substrate utilization efficiency (Table 3).

The kinetic parameters measured for the isomerase activity of purified β HSD toward 5-androstene-3,17-dione and the cofactor NADH, in the presence and absence of purified Cyt- b_5 , showed that neither the K_m values ($K_{m,app,5\text{-androstene-3,17-dione}}$, $K_{m,NADH}$) nor the V_{max} value are influenced by Cyt- b_5 (Table 3 and Fig. 5C, D).

Interaction between β HSD and Cyt- b_5 in COS-1 cells

Colocalization and FRET analysis

The subcellular localization of β HSD and Cyt- b_5 was investigated by coexpressing β HSD-eCFP and Cyt- b_5 -eYFP in COS-1 cells. The fluorescence detected (Fig. 6) clearly confirmed that both fusion proteins are localized to the ER (data not shown). The interaction

TABLE 3. Comparison of the kinetic parameters for the dehydrogenase and isomerase activities of purified β HSD in the presence and absence of purified Cyt- b_5

Activity and treatment	Parameter			
	K_m (μ M)	V_{max} (nmol \cdot min $^{-1}\cdot$ mg $^{-1}$)	K_{cat} (min $^{-1}$)	K_{cat}/K_m (min $^{-1}\cdot\mu$ M $^{-1}$)
Dehydrogenases				
DHEA ^a				
–Cyt- b_5	1.2 ± 0.2	51.9 ± 1.6	2.2 ± 0.03	1.8 ± 0.15
+Cyt- b_5	1.1 ± 0.3^{ns}	$62.5 \pm 2.9^*$	$2.5 \pm 0.05^*$	$2.4 \pm 0.3^*$
NAD ⁺				
–Cyt- b_5	25.4 ± 4.9	59.2 ± 4.1	2.4 ± 0.1	0.1 ± 0.01
+Cyt- b_5	$7.7 \pm 3.5^{***}$	59.7 ± 3.6^{ns}	2.5 ± 0.09^{ns}	$0.4 \pm 0.1^{***}$
Isomerases				
5-Androstene-3,17-dione ^a				
–Cyt- b_5	39.2 ± 8.0	221.9 ± 15.6	9.3 ± 0.4	0.2 ± 0.02
+Cyt- b_5	40.6 ± 7.8^{ns}	232.7 ± 15.7^{ns}	9.8 ± 0.4^{ns}	0.2 ± 0.02^{ns}
NADH				
–Cyt- b_5	4.1 ± 0.8	263.3 ± 12.8	11.1 ± 0.3	2.8 ± 0.3
+Cyt- b_5	3.4 ± 0.8^{ns}	266 ± 13.7^{ns}	11.2 ± 0.3^{ns}	3.4 ± 0.5^{ns}

K_m , V_{max} , K_{cat} , and substrate utilization efficiency (K_{cat}/K_m) values are expressed as means \pm SE of triplicate experiments. Value for each parameter obtained in the absence of Cyt- b_5 is compared to that obtained in the presence of Cyt- b_5 using an unpaired t test ($n=3$). ^{ns} $P > 0.05$; ^{*} $P < 0.05$; ^{**} $P < 0.01$; ^{***} $P < 0.001$. ^aData are representative of apparent values.

between β HSD and Cyt- b_5 was subsequently investigated by FRET. β HSD-eCFP was coexpressed with either CYP17A1-eYFP, β HSD-eYFP, or Cyt- b_5 -eYFP in COS-1 cells and screened for FRET. A relatively weak FRET signal, only marginally higher than that of the background signal, was observed in cells coexpressing β HSD-eCFP and CYP17A1-eYFP, a microsomal enzyme, which served as a negative control ($-V_e$). Cells expressing a CFP-YFP fusion protein served as a positive control ($+V_e$) for FRET. A significant FRET signal, ≈ 2 -fold greater than that of the positive control, was detected in cells coexpressing β HSD-eCFP and Cyt- b_5 -eYFP (Fig. 7). These data show that β HSD and Cyt- b_5 interact directly with each other in live cells, confirming allosteric interaction between the two proteins. Inter-

estingly, a significant ($P < 0.001$) FRET signal was observed in cells coexpressing β HSD-eCFP and β HSD-eYFP, though this signal was significantly smaller than that obtained for β HSD and Cyt- b_5 . Nevertheless, these data suggest possible homomeric complex formation between β HSD, as has been observed for other microsomal steroidogenic enzymes (23–26), although further investigation is required.

Influence of fluorescent tags on the interaction between β HSD and Cyt- b_5

It is possible that the fluorescent proteins fused to β HSD and Cyt- b_5 may influence the activities of the respective proteins and subsequently the augmentation

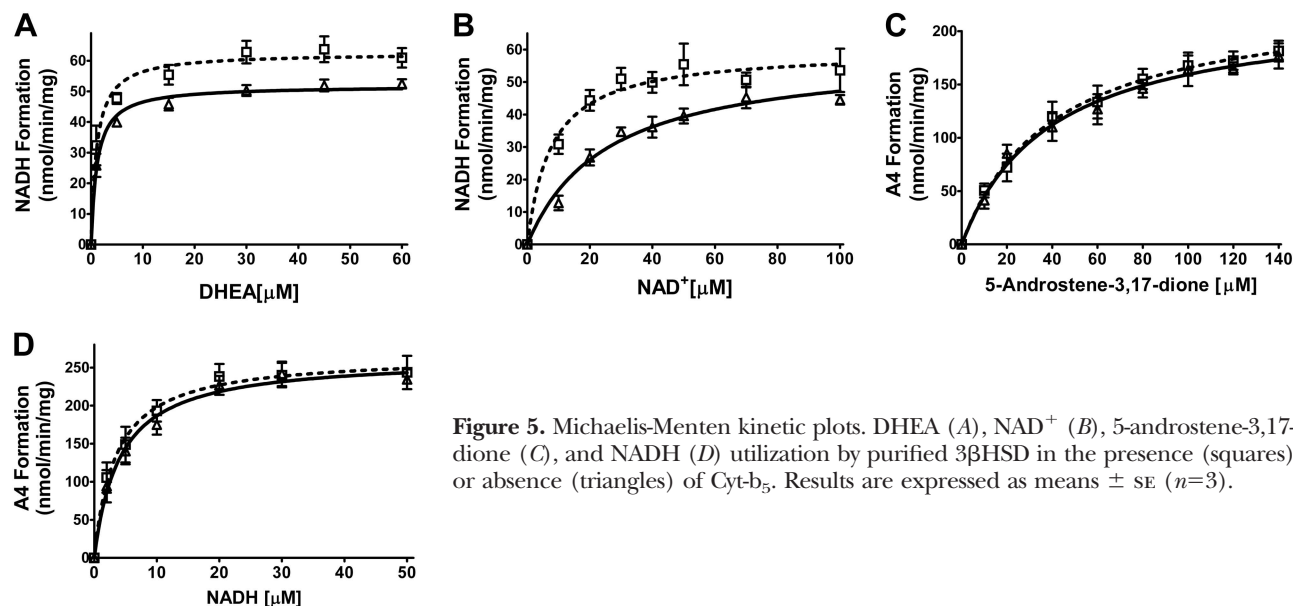


Figure 5. Michaelis-Menten kinetic plots. DHEA (A), NAD⁺ (B), 5-androstene-3,17-dione (C), and NADH (D) utilization by purified β HSD in the presence (squares) or absence (triangles) of Cyt- b_5 . Results are expressed as means \pm SE ($n=3$).

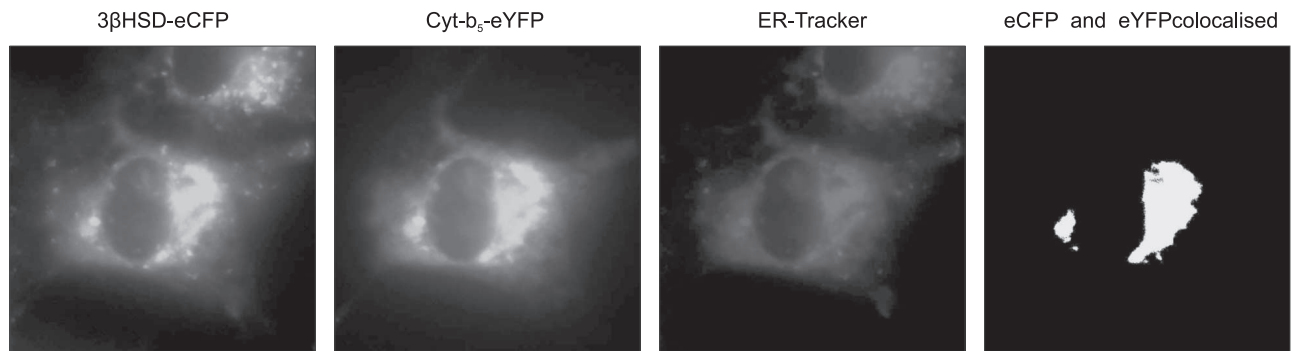


Figure 6. Colocalization of fusion proteins in the ER of COS-1 cells. 3βHSD-eCFP fluorescence (A), Cyt-b₅-eYFP fluorescence (B), ER-Tracker fluorescence (C), and eCFP and eYFP signal (D).

of 3βHSD by Cyt-b₅ (12). The catalytic activity of 3βHSD and 3βHSD-eCFP was therefore compared by expressing both the proteins with either Cyt-b₅ or Cyt-b₅-eYFP in COS-1 cells. Cells coexpressing 3βHSD and Cyt-b₅ converted ≈25% more DHEA to A4 ($P < 0.01$) after 2 h when compared to cells expressing only 3βHSD (Fig. 8), which is in agreement with our previous findings (12). Similarly, the conversion of DHEA to A4 by cells coexpressing either 3βHSD-eCFP and Cyt-b₅; 3βHSD and Cyt-b₅-eYFP or 3βHSD-eCFP and Cyt-b₅-eYFP was significantly greater than that of cells only expressing 3βHSD and not significantly different from cells expressing untagged 3βHSD and Cyt-b₅. These results clearly indicate that the addition of the fluorescent tags to either 3βHSD or Cyt-b₅ had no effect on the stimulatory interaction between these two proteins.

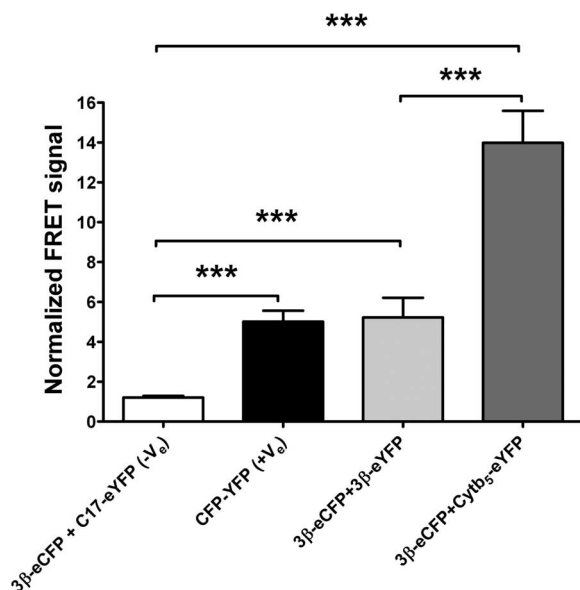


Figure 7. Normalized FRET signal in COS-1 cells expressing fusion proteins. Background subtraction was carried out using an area where no cells were present; negative control, -V_e; positive control, +V_e. Normalized FRET signal was compared individually for each coexpression using an unpaired *t* test. Results are expressed as means ± SE (*n*=3). *** $P < 0.001$.

DISCUSSION

We previously reported that Cyt-b₅ augments 3βHSD activity in COS-1 cells, as well as in ovine adrenal microsomal preparations. We observed that while Cyt-b₅ had no effect on the substrate affinity of the enzyme, it resulted in an apparent overall increase in reaction rate. As a result, we hypothesized that the augmentation of 3βHSD activity by Cyt-b₅ was most likely allosteric in nature, resulting in a increased affinity for the cofactor NAD⁺, thereby facilitating the observed increase in the rate of catalysis (12). In this study, the influence of Cyt-b₅ on the individual dehydrogenase and isomerase reactions of 3βHSD was investigated. To this end, unmodified caprine 3βHSD was overexpressed using a baculovirus expression system. SDS-PAGE analysis revealed the successful purification of a ≈42 kDa protein (Fig. 2), which was confirmed to be 3βHSD by mass spectrometry (Table 2).

Previously, we reported that the level of 3βHSD stimulation by Cyt-b₅ was dependent on the amount of Cyt-b₅ added to a microsomal preparation (12). Therefore, the activation of purified 3βHSD by Cyt-b₅ was initially assessed at various ratios of purified Cyt-b₅ to 3βHSD. The results showed that optimum stimulation was achieved at a ratio of 1:1 (Fig. 4). However, when the ratio was adjusted beyond 1:1, a significant ($P < 0.05$) reduction in catalytic stimulation was observed. These results exhibited a similar trend as those observed in adrenal microsomes (12). It is possible that the decline in stimulation at higher ratios of Cyt-b₅ may be as a result of Cyt-b₅-forming aggregates, known to occur at higher concentrations of Cyt-b₅ (27).

Following the optimization of the ratio required for maximal stimulation, kinetic parameters were determined for both the dehydrogenase and isomerase reactions (Table 3 and Fig. 5). Cyt-b₅ had no observable effect on the $K_{m,DHEA}$ value, while significantly ($P < 0.05$) increasing the $V_{max,app}$ value toward DHEA during the first dehydrogenation reaction (Table 3 and Fig. 5A). These results correlated with the results previously obtained in COS-1 cells, confirming that the stimulatory effect of Cyt-b₅ has no influence on the ability of 3βHSD to bind steroid substrate (12). The results further demonstrated

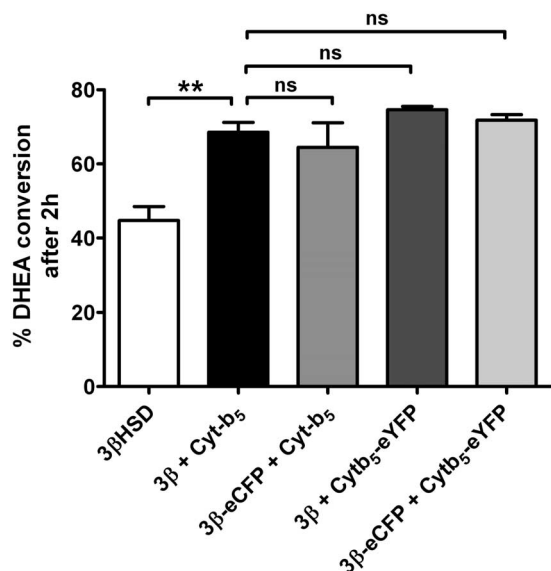


Figure 8. DHEA metabolism by 3βHSD in COS-1 cells after 2 h. 3βHSD and 3βHSD-eCFP coexpressed with either pCI-neo, Cyt-b₅, or Cyt-b₅-eYFP. Percentage of DHEA conversion after 2 h is compared individually for each coexpression using an unpaired *t* test. Results are expressed as means ± SE (*n*=3). ns, not significant (*P*>0.05). ***P* < 0.01.

that the presence of Cyt-b₅ stimulated the dehydrogenase reaction, as significant (*P*<0.05) increases were observed in the turnover rate $K_{cat,app}$ and substrate utilization efficiency, $K_{cat,app}/K_{m,app}$ (Table 3). In addition, the presence of Cyt-b₅ significantly increased the affinity of 3βHSD for the dehydrogenase cofactor NAD⁺, as Cyt-b₅ decreased the K_{m,NAD^+} value ≈3.5-fold and significantly (*P*<0.001) increased the efficiency with which 3βHSD utilizes NAD⁺ (Table 3 and Fig. 5B).

The kinetic data obtained for the second isomerase reaction revealed the $V_{max,app}$ value during the utilization of 5-androstene-3,17-dione to be ≈4-fold greater than that for the initial dehydrogenase reaction during the utilization of DHEA (Table 3). Similarly, it has previously been shown that the isomerase activity of human 3βHSD is ≈10-fold greater than the dehydrogenase activity (16, 18, 19). In this study, Cyt-b₅ had no significant influence on the apparent kinetic parameters determined for 5-androstene-3,17-dione as substrate or the kinetic parameters of NADH as cofactor during the second isomerase reaction (Table 3 and Fig. 5C, D).

Taken together, these data indicate that Cyt-b₅ increases the efficiency of the “rate-limiting” dehydrogenase reaction of 3βHSD by increasing the affinity of the enzyme toward NAD⁺, while having no significant effect on the second isomerase reaction. As the isomerase activity was shown to be ≈4-fold greater than the dehydrogenase activity, such stimulation of the dehydrogenase reaction would account for the observed increase in $V_{max,app}$ of the enzyme (12). While the decrease in K_{m,NAD^+} cannot result in an actual increase in the V_{max} of the enzyme, under nonsaturating conditions of NAD⁺, such as those in living cells, an in-

creased affinity for the cofactor NAD⁺ results in an increase in $V_{max,app}$.

In vivo, the concentration of free cofactor exceeds that of steroid hormones by many orders of magnitude (28). The researchers suggested that the activity of HSDs may thus be modulated to a greater extent by the abundance of cofactor and by the relative affinity of these enzymes toward their cofactors rather than steroid concentration (28). It is therefore plausible that the activity of 3βHSD toward its respective steroid substrates may be altered or augmented by Cyt-b₅, influencing the affinity of the enzyme for NAD⁺.

Traditionally, Cyt-b₅ is believed to augment the activity of specific CYPs *via* direct electron transfer. It was, however, demonstrated by Auchus *et al.* (24) that *apo*-b₅ was able to stimulate the 17,20-lyase activity of CYP17A1, prompting the researchers to propose an allosteric mechanism of augmentation. In addition, other studies have also implicated Cyt-b₅ as playing an allosteric role in the catalysis of various other CYP enzymes, which include CYP3A4, CYP3A5, and CYP2A6 (29, 30). Unlike the CYPs, 3βHSD does not require the input of electrons from an external electron donor to perform its catalytic function. It is therefore likely that Cyt-b₅ would augment 3βHSD activity *via* an allosteric mechanism.

We therefore investigated the interaction of these two proteins using FRET. 3βHSD-eCFP and Cyt-b₅-eYFP were both localized in the ER membrane of COS-1 cells (Fig. 6), and a strong FRET signal was obtained when both proteins were coexpressed, confirming that the proteins do interact in live cells (Fig. 7). Furthermore, tagging the proteins with either eCFP or eYFP had no effect on the stimulatory influence of Cyt-b₅ on 3βHSD (Fig. 8). Taken together, these data, together with our previous observation that *apo*-b₅ stimulated 3βHSD activity, confirm that the augmentation of 3βHSD activity by Cyt-b₅ is allosteric in nature and independent of direct electron transfer (12). Homology modeling of human type I 3βHSD has shown the dehydrogenase and isomerase domains to be linked by a shared Rossmann-fold coenzyme domain (6) that may be separate from the substrate domain. The data therefore clearly indicate that the allosteric interaction of Cyt-b₅ selectively influences NAD⁺ binding without effecting substrate binding.

In adrenal steroidogenesis, the production of A4, a vital precursor of active sex steroids, requires the coexpression of 3βHSD, CYP17A1, and Cyt-b₅ (31). While significant levels of circulating A4 are believed to originate from the adrenal cortex (32–34), the expression of 3βHSD is low in the zona reticularis (ZR), which specifically expresses CYP17A1 and Cyt-b₅ to produce DHEA. Conversely, 3βHSD is expressed in the zona fasciculata (ZF) and zona glomerulosa (ZG), characterized by low levels of Cyt-b₅ (35, 36). However, Nakamura *et al.* (37) recently identified a layer of adrenocortical cells located between the ZF and ZR that express 3βHSD, CYP17A1, and Cyt-b₅ and as such have been postulated to be the primary site of A4 production in the adrenal. While Cyt-b₅ is vital in the stimulation of the 17,20-lyase

reaction of CYP17A1, the stimulation of 3β HSD by Cyt- b_5 may be equally relevant in the production of A4 by these cells, particularly as 17-OHPROG is a poor substrate for the 17,20-lyase reaction of human and ovine CYP17A1 (2, 7, 20, 37). Since the stimulation of 3β HSD activity by Cyt- b_5 is only between 15 and 25% (12), a relatively small increase in comparison to the augmentation of the 17,20-lyase activity of CYP17A1 by Cyt- b_5 (\approx 10-fold; ref. 11), the physiological relevance thereof may thus be questionable. However, a recently published mathematical model simulating the network of reactions catalyzed by 3β HSD and CYP17A1 revealed that small increases in 3β HSD activity result in a substantial increase in A4 production, with optimal A4 production being reached within a comparatively small activity window. Furthermore, the model revealed that increasing the activity of 3β HSD beyond a certain point paradoxically reduces A4 production, as significantly more PREG and 17OHPREG are converted to PROG and 17OHPROG, neither of which are precursors for A4 (38). These data therefore demonstrate that a small increase in 3β HSD activity has the potential to increase A4 production substantially, highlighting the physiological relevance of the augmentation of 3β HSD activity by Cyt- b_5 in adrenal A4 production.

CONCLUSIONS

The data presented in this study provide clear evidence that an allosteric interaction between 3β HSD and Cyt- b_5 increases the affinity of 3β HSD for NAD^+ , resulting in an increase in the efficiency of the “rate-limiting” dehydrogenase reaction and as a result, an increase in the overall efficiency of the enzyme. This represents the first documentation of Cyt- b_5 influencing cofactor binding in any member of the HSDs. **[F]**

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6.2 Conclusion

The data presented in this chapter clearly indicates that the presence of Cyt-b₅ substantially increased the affinity of 3 β HSD for NAD⁺, resulting in an increase in the apparent V_{max} of the "rate limiting" dehydrogenase reaction leading to an increase in the overall rate of catalysis. Furthermore, FRET analysis of COS-1 cells coexpressing 3 β HSD-eCFP and Cyt-b₅-eYFP fusion proteins strongly suggests an allosteric interaction between these proteins in live cells. Therefore these observations validated our previous hypothesis and represent the first documentation of Cyt-b₅ influencing co-factor binding in any member of the hydroxysteroid dehydrogenases.

CHAPTER 7

GENERAL DISCUSSION

In the adrenals, 3 β HSD activity plays a key role in the biosynthesis of all classes of adrenocortical hormones. In addition, 3 β HSD also competes with CYP17 for Δ^5 steroid intermediates at key branch points during adrenal steroidogenesis. The catalytic activity and substrate specificity of this key steroidogenic enzyme are therefore crucial in determining the flux of steroid intermediates through the steroid hormone biosynthesis pathway (Conley and Bird, 1997; Penning, 1997; Payne and Hales, 2004, Miller and Auchus, 2011). Nonetheless, little experimental data are currently available describing the influence that varying levels of 3 β HSD activity have on the competition between 3 β HSD and CYP17 and how fluctuations in the outcome of this competition ultimately impacts the biosynthesis of active steroid hormones. The aim of this thesis was therefore to investigate how variations in 3 β HSD activity influence the flux of steroid intermediates through the various steroidogenic pathways and to identify and characterize specific factors that influence this activity. In order to achieve these aims a relevant experimental model needed to be employed. The South African Angora goat provided an excellent model as these animals exhibit a known steroidogenic deficiency that may result from altered 3 β HSD activity levels.

As described in chapter 4, the influence of 3 β HSD activity on adrenal steroidogenesis was investigated by characterizing and comparing Angora 3 β HSD to ovine 3 β HSD. Angora and ovine adrenal cortex homogenates were prepared from multiple adrenal glands collected from animals which varied in age and sex. Total mRNA was isolated from these homogenates and the cDNA sequences encoding Angora and ovine 3 β HSD were amplified from the mRNA through RT-PCR and cloned into a mammalian expression vector. Direct DNA sequence analysis revealed 97% sequence identity between the Angora (GenBank accession no. EF524065) and ovine adrenal 3 β HSD (GenBank accession no. FJ007375) cDNA sequences, which represents the only known form of the enzyme in both species. Translation of these sequences identified five differences in the primary structure of which two are located in an NH₂-terminal coenzyme binding domain consisting of a β - α - β - α - β motif common to all members of the short-chain dehydrogenase/reductase (SDR) family of enzymes (Jörnvall et al., 1995; Simard et al., 2005; Kavanagh et al., 2008). Furthermore, another substitution was found to be located adjacent to a catalytic motif, Tyr¹⁵⁴-X-X-X-Lys¹⁵⁸, present in the primary structure of all 3 β HSDs (Luu-The et al., 1989; Simard et al., 2005). This motif has been implicated in

the dehydrogenase activity of 3 β HSD whilst the residue at position 156 has been shown to play an important role in the binding of steroid substrate (Thomas et al., 2002). Therefore, these differences in primary structure can lead to differences in substrate binding, co-factor binding and dehydrogenase activity between the enzymes for which they encode.

Both Angora and ovine 3 β HSD were subsequently expressed in COS-1 cells and the activity of each enzyme assayed towards PREG, 17-OHPREG and DHEA in order to determine how these differences in primary structure influence 3 β HSD activity. Similar initial reaction rates were observed for both Angora and ovine 3 β HSD during the conversion of PREG. However, the initial reaction rate of ovine 3 β HSD during the conversion of 17-OHPREG was significantly greater than that of the Angora enzyme, whilst that of Angora 3 β HSD towards DHEA was significantly greater than that of the ovine enzyme. The apparent K_m and V_{max} values of each enzyme, towards the respective steroid substrates, were subsequently determined and found to reflect the initial reaction rates.

As previously shown by Engelbrecht and Swart (2000) the majority of steroid intermediates are committed to the Δ^5 pathway during adrenal steroidogenesis in the South African Angora goat. Consequently, 3 β HSD activity becomes essential for the production of cortisol by converting 17-OHPREG to the cortisol precursor 17-OHPROG. However, the significantly lower activity of Angora 3 β HSD towards 17-OHPREG could impair the adequate formation of 17-OHPROG, co-implicating 3 β HSD in causing hypocortisolism in the South African Angora goat.

To ascertain how differences in 3 β HSD activity influence the flux of steroid intermediates through the steroidogenic pathways and ultimately cortisol production, Angora and ovine 3 β HSD were individually coexpressed with Angora CYP17 in COS-1 cells and assayed with PREG and 17-OHPREG as substrates. The results revealed a significant difference in the profile of steroid metabolites produced by cells expressing Angora 3 β HSD compared to those expressing ovine 3 β HSD. Following the metabolism of PREG, cells expressing Angora 3 β HSD produced significantly less 17-OHPROG whilst producing significantly more PROG and A4 compared to cells expressing ovine 3 β HSD. Similarly, cells expressing Angora 3 β HSD produced significantly less 17-OHPROG and more A4 following the metabolism of 17-OHPREG. Therefore, as the majority of steroid intermediates are committed to the Δ^5 pathway in the Angora, the lower catalytic activity of Angora 3 β HSD towards 17-OHPREG results in less 17-OHPROG being synthesized, implicating 3 β HSD as an additional factor in causing hypocortisolism in these animals.

It is known that 3 β HSD deficiency in humans result in a variety of clinical disorders due to the insufficient synthesis of hormones such as cortisol, aldosterone and testosterone, with the severity of the symptoms depending on the degree to which 3 β HSD activity is impaired (Moisan et al., 1999;

Pang, 2001; Simard et al., 2005). However, the manner in which variations in 3β HSD activity alter the biosynthesis of these hormones is not fully characterized. Using the South African Angora goat as model, the data presented in chapter 4 suggests that the differences in 3β HSD activity observed between these two species significantly impacts the competition between 3β HSD and CYP17 for PREG and 17-OHPREG. This in turn alters the flux of steroid intermediates through the steroidogenic pathways leading to a significant reduction in the synthesis of the cortisol precursor 17-OHPROG. These findings implicate 3β HSD as an additional factor in causing hypocortisolism and illustrate how variations in 3β HSD activity alters the flux of steroid intermediates in order to contribute to a known steroidogenic deficiency.

In addition to variations in 3β HSD activity, caused by differences in primary structure, the competition between 3β HSD and CYP17 for 17-OHPREG is also influenced by Cyt-b₅. Cyt-b₅ selectively stimulates the lyase activity of CYP17 towards 17-OHPREG, whilst resulting in a comparatively small enhancement in activity towards 17-OHPROG, leading to a substantial increase in C19 steroid production (Katagiri et al., 1982; Katagiri et al., 1995; Auchus et al., 1998). As 3β HSD, CYP17 and Cyt-b₅ are coexpressed in the Angora adrenal, Cyt-b₅ may further contribute to this condition of hypocortisolism (Storbeck et al., 2007). Consequently, the outcome of the competition between 3β HSD and CYP17 during the metabolism of 17-OHPREG in the presence of Cyt-b₅ was investigated. In COS-1 cells, Angora and ovine 3β HSD were each coexpressed with Angora CYP17 in the presence and absence of overexpressed Angora Cyt-b₅ and assayed with 17-OHPREG as substrate.

As Cyt-b₅ selectively stimulates the lyase activity of CYP17 towards 17-OHPREG it was expected that the presence of overexpressed Cyt-b₅ would result in reduced 17-OHPROG biosynthesis and increased A4 synthesis. In addition, it was expected that cells expressing Angora 3β HSD would produced lower levels of 17-OHPROG compared to cells expressing ovine 3β HSD due to the lower activity of Angora 3β HSD towards 17-OHPREG. As expected, the presence of overexpressed Cyt-b₅ substantially increased A4 production at the expense of 17-OHPROG production. However, the presence of overexpressed Cyt-b₅ caused cells expressing Angora 3β HSD to produced significantly higher levels of 17-OHPROG compared to cells expressing ovine 3β HSD, which sharply contrasted the results obtained in the absence of overexpressed Cyt-b₅.

In the Angora, the substrate for the 17,20 lyase reaction is almost exclusively 17-OHPREG (Storbeck et al., 2007). In addition, Cyt-b₅ results in a comparatively small enhancement of CYP17 lyase activity towards 17-OHPROG (Storbeck et al., 2007), indicating that an increase in A4 production in the presence of overexpressed Cyt-b₅ occurs via DHEA and not 17-OHPROG. Furthermore, as the lyase activity of CYP17 is augmented equally in cells expressing either Angora or

ovine 3 β HSD, the presence of overexpressed Cyt-b₅ could not account for the differences observed in 17-OHPROG production between the two species. It was apparent that these data could not be accommodated within the bounds of our current understanding of how Cyt-b₅ influences adrenal steroidogenesis, alluding to the possibility that Cyt-b₅ may affect steroidogenesis, not only by enhancing lyase activity of CYP17, but also by exerting an influence on 3 β HSD activity. Although no evidence was available in the literature that would suggest an interaction between Cyt-b₅ and 3 β HSD, differential augmentation by Cyt-b₅ of Angora and ovine 3 β HSD activity towards 17-OHPREG and DHEA, could account for the altered steroid profile observed. The augmentation of 3 β HSD activity by Cyt-b₅ was subsequently investigated. It was also important to ascertain whether the augmentation was specific to 3 β HSD, or an artifact of the COS-1 cell expression system.

As described in chapter 5, Cyt-b₅ was coexpressed with both Angora and ovine 3 β HSD in COS-1 cells and the catalytic activity of each enzyme assayed towards PREG, 17-OHPREG and DHEA. The results showed Cyt-b₅, coexpressed with either Angora or ovine 3 β HSD, significantly increased the conversion of all three steroid substrates. In addition, the degree of stimulation was found to vary between the two species and between the respective substrates, indicating that augmentation occurs in a substrate and species specific manner.

Since 3 β HSD and Cyt-b₅ are both localized to the ER membrane coexpression with Cyt-b₅ might alter the ER membrane environment to such an extent that 3 β HSD becomes more accessible to steroid substrates and co-factors, resulting in non-specific stimulation of 3 β HSD activity independent of Cyt-b₅. The validity of these results were therefore evaluated by coexpressing 3 β HSD with another microsomal enzyme, 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), and a truncated form of Cyt-b₅ (trunc-b₅) which would not associate with the ER membrane as it does not contain a membrane binding domain. The presence of 11 β HSD2 was subsequently shown to have no significant influence on 3 β HSD activity whilst the cytosolic truncated form of Cyt-b₅ was found to stimulate 3 β HSD activity to the same extent as holo-b₅. Thus, the augmentation of 3 β HSD activity by Cyt-b₅ could not be attributed to non-specific membrane effects.

The validity of this augmentation was further evaluated using an adrenal microsomal preparation. This provided a tool with which to study the influence of Cyt-b₅ on 3 β HSD activity in a complex membrane environment containing 3 β HSD and Cyt-b₅ as well as numerous other membrane bound proteins. Results showed the addition of anti-Cyt-b₅ IgG to significantly reduced 3 β HSD activity towards PREG while addition of purified Cyt-b₅ significantly increased the activity of 3 β HSD towards PREG and DHEA. These data confirmed the augmentation of 3 β HSD activity by Cyt-b₅ in a native

membrane environment and further illustrated that the augmentation observed in COS-1 cells is specific to Cyt-b₅ and not an artifact of the COS-1 cell expression system.

Cyt-b₅ is a ubiquitous electron transfer hemoprotein known to affect the substrate metabolism of various cytochromes P450. However, the mechanism through which Cyt-b₅ affects P450 catalysis has remained controversial. Traditionally, Cyt-b₅ is believed to augment the activity of cytochromes P450 via direct electron transfer. Initial studies investigating this mechanism suggested that Cyt-b₅ augments P450 catalysis by acting as an alternative redox partner, resulting in the faster input of the second electron, thereby reducing the spontaneous decay of the oxycytochrome P450 complex allowing for more product to form (Hildebrandt and Estabrook, 1971; Schenkman and Jansson, 2003; Akhtar et al., 2005). More recently, a second electron transfer model has been proposed that suggests an electron transfer and effector (allosteric) function for Cyt-b₅ during CYP2B4 catalysis. According to this hypothesis, during the transfer of the second electron from Cyt-b₅ to CYP2B4, Cyt-b₅ binds to CYP2B4 causing conformational changes in the active site, allowing the oxyferrous specie to be formed more rapidly (Bridges et al., 1998; Zang et al., 2003, 2007). However, a growing body of evidence now suggests that Cyt-b₅ also acts as an allosteric modulator without directly participating in electron transfer (Morgan and Coon, 1984; Yamazaki et al., 1996; Yamazaki et al., 2002). In the case of CYP17, Auchus et al (1998) demonstrated that CYP17 lyase activity could be stimulated by varying the molar ratios of Cyt-b₅:CYP17 whilst maintaining a fixed POR concentration. Furthermore, apo-b₅ was also shown to stimulate CYP17 lyase activity, leading the authors to hypothesize that Cyt-b₅ modifies the CYP17·POR complex allosterically, thereby facilitating more efficient electron transfer during catalysis.

Unlike, the cytochromes P450, 3βHSD does not require electrons from an external electron donor to perform its catalytic function (Thomas et al., 1989, 1995, 2003). It therefore seems unlikely that electron transfer plays a role in the augmentation of 3βHSD activity by Cyt-b₅. Thus, the mechanism through which Cyt-b₅ augments 3βHSD activity was investigated. 3βHSD was coexpressed with either holo- or apo-b₅ (wild type Cyt-b₅ void of heme moiety) in COS-1 cells and assayed with PREG as substrate. Both holo- and apo-b₅ were demonstrated to stimulate 3βHSD activity, suggesting that stimulation occurs via an allosteric mechanism, as apo-b₅ is unable to participate in electron transfer reactions. This mechanism was further investigated by determining kinetic constants for 3βHSD in the presence and absence of Cyt-b₅. The results revealed Cyt-b₅ to have no influence on the apparent K_m values whilst significantly increasing the apparent V_{max} values towards each of the three steroid substrates evaluated. The unchanged apparent K_m values suggested that an allosteric interaction between 3βHSD and Cyt-b₅ is unlikely to result in significant conformational changes in the active site.

Still, subtle changes brought about by this interaction may influence co-factor binding. Indeed, homology modeling of human type I 3 β HSD has shown the dehydrogenase and isomerase domains to be linked by a separate shared co-enzyme domain (Thomas et al., 2003). Thus, it seems plausible that the binding of NAD⁺ may be affected resulting in the stimulation of the dehydrogenase activity without influencing steroid binding.

The influence of Cyt-b₅ on co-factor binding was subsequently evaluated by theoretical computer modeling. A simplified irreversible bi-substrate rate equation was used to construct a model in order to simulate the effect that alterations in K_{NAD^+} value would have on the catalytic activity of 3 β HSD. These simulations demonstrated that the apparent V_{max} value could be increased by decreasing the K_{NAD^+} value without altering the K_m value for the steroid substrate, corresponding to the kinetic data generated in COS-1 cells. According to these data we hypothesized that an allosteric interaction between 3 β HSD and Cyt-b₅ results in an increase in the affinity of 3 β HSD for NAD⁺, stimulating the dehydrogenase reaction, resulting in an increase in the overall catalytic activity of the enzyme. As the rate of the second isomerase reaction has been shown to be substantially greater than that of the first dehydrogenase reaction (Thomas et al., 1989, 1995, 2003) an increase in the rate of the dehydrogenase reaction would account for the increase observed in the overall rate of the enzyme.

3 β HSD catalyzes the conversion of Δ^5 steroids to their corresponding Δ^4 products via sequential dehydrogenase and isomerase reactions. In the first dehydrogenase reaction, NAD⁺ is reduced to NADH with the formation of a Δ^{5-3} keto steroid intermediate which, together with NADH, remains bound to the enzyme. NADH subsequently induces conformational changes in the enzyme resulting in the isomerization of the intermediate to form the corresponding Δ^4 steroid product (Thomas et al., 1989, 1995, 2003). Due to the nature of the COS-1 cell and microsomal systems, the results discussed thus far only gave an indication of the overall effect of Cyt-b₅ on 3 β HSD activity but provided no indication as to the influence of Cyt-b₅ on the individual activities of this enzyme. Further investigation therefore focused on the influence of Cyt-b₅ on the individual dehydrogenase and isomerase activities of 3 β HSD as well as co-factor binding using purified enzyme.

As described in chapter 6, 3 β HSD was overexpressed in SF-9 cells using a baculovirus expression system and purified with a detergent based enzyme purification method. Following purification, both substrate and co-factor kinetics were independently determined for both the dehydrogenase and isomerase activities in the presence and absence of purified Cyt-b₅. The results showed the presence of Cyt-b₅ to have no significant influence on the steroid substrate kinetics for both the dehydrogenase and isomerase reactions. In addition, the apparent K_m value of purified Angora 3 β HSD towards DHEA (1.2 ± 0.2 SEM) was found to be almost identical to that determined in COS-1

cells (1.3 ± 0.1 SEM), confirming the validity of these results and providing additional evidence that Cyt-b₅ does not influence steroid binding. Furthermore, Cyt-b₅ significantly increased the apparent V_{max} of the dehydrogenase reaction whilst resulting in a ≈ 3.5 -fold decrease in the K_m value of 3 β HSD towards NAD⁺. In contrast, the presence of Cyt-b₅ had no significant influence on the kinetic parameters of the isomerase reaction. Moreover, the kinetic data also revealed the apparent V_{max} value for the isomerase reaction during the utilization of 5-androstene-3,17-dione to be ≈ 4 -fold greater than that for the initial dehydrogenase reaction during the conversion of DHEA. This confirmed that the overall reaction rate is dependant on the rate of the first dehydrogenase reaction rather than that of the second isomerase reaction.

Taken together, these data indicated that Cyt-b₅ stimulates the initial "rate-limiting" dehydrogenase reaction of 3 β HSD by increasing the affinity of the enzyme towards NAD⁺, whilst having no significant effect on the second isomerase reaction. These results therefore confirmed our previous hypothesis stating that the augmentation of 3 β HSD activity by Cyt-b₅ is due to a decrease in the K_{NAD^+} value leading to an overall increase in rate of the enzyme.

This stimulatory interaction between 3 β HSD and Cyt-b₅ is most likely allosteric in nature as apo-b₅ was shown to also stimulate 3 β HSD activity. Thus, the interaction between these proteins was investigated in the ER membrane of COS-1 cells using fusion proteins. 3 β HSD-eCFP and Cyt-b₅-eYFP were coexpressed in COS-1 cells and analyzed for FRET. A strong FRET signal was obtained when both 3 β HSD-eCFP and Cyt-b₅-eYFP were coexpressed, confirming that these proteins do interact in live cells which, together with the data obtained with apo-b₅, clearly indicated that this interaction is allosteric in nature. The exact mechanism through which this interaction occurs is, however, as yet uncertain.

In the case of CYP17, it is believed that Cyt-b₅ interaction occurs via ionic interactions through positive surface charges on CYP17 interacting with negative charges on Cyt-b₅. Indeed, CYP17 mutant enzymes containing either an Arg347His or Arg358Gln mutation showed $\approx 5\%$ lyase activity when expressed in COS-1 cells in the presence of Cyt-b₅. In addition, substitution of these Arg residues with Lys residues did not influence the Cyt-b₅ dependant lyase activity of CYP17. Furthermore, substitution of Arg449 with an Ala residue abolishes Cyt-b₅ dependant lyase activity (Geller et al., 1999; Yamazaki et al., 2002). In addition, Cyt-b₅ mutants containing Glu48Gly and Glu49Gly substitutions exhibited a decreased ability to stimulate CYP17 lyase activity (Naffin-Olivos and Auchus, 2006). Thus, it is possible that ionic interactions between 3 β HSD and Cyt-b₅ may play a role in the stimulatory interaction between these proteins. As the truncated version of Cyt-b₅ (1-80 AA) was shown to stimulate 3 β HSD activity to the same extent as holo-b₅, it can be assumed that the regions responsible

for interacting with 3 β HSD are located on the hydrophilic region of the protein. This seems plausible as removal of the heme moiety, known to result in substantial conformational changes in the head domain (Pfeil, 1993; Storch and Daggett, 1996), reduced the ability of Cyt-b₅ to stimulate 3 β HSD activity, suggesting that the hydrophilic region of Cyt-b₅ may play a role in the interaction with 3 β HSD (Goosen et al., 2011). Furthermore, the stimulatory interaction between 3 β HSD and Cyt-b₅ may also be influenced by the aggregation state of Cyt-b₅. Lombard et al (2002) previously showed the ability of Cyt-b₅ to aggregate and that these multimeric complexes were likely to be present *in vivo*. In chapters 5 and 6 of this study we showed that the addition of purified Cyt-b₅ in varying ratios, to either a microsomal preparation or pure enzyme, stimulated 3 β HSD activity up to a certain point. However, if the level of Cyt-b₅ added was increased above that of the optimum, stimulation of 3 β HSD activity was significantly reduced. This phenomenon may be attributed to Cyt-b₅ forming aggregates so that the regions that interact with 3 β HSD become shielded. Nevertheless, more research is needed to characterize the mechanism through which 3 β HSD and Cyt-b₅ interaction occurs as well as the factors which influence this interaction.

The data presented in this study clearly indicate that an allosteric interaction between 3 β HSD and Cyt-b₅ increases the affinity of 3 β HSD for NAD⁺, resulting in an increase in the overall rate of catalysis. This represents a novel discovery in the field of steroid biochemistry as the augmentation of 3 β HSD activity by Cyt-b₅ was previously undocumented. Still, the physiological relevance of such an augmentation of 3 β HSD activity by Cyt-b₅ during adrenal steroidogenesis in humans and other mammals seemed unclear due to the functional zonation of the adrenal cortex. In humans, immunohistochemical analysis of the adrenal cortex has shown the expression of 3 β HSD and Cyt-b₅ to vary between the three cortical zones during development. Suzuki et al (2000) showed 3 β HSD expression to remain relatively constant in the zona fasciculata and glomerulosa but substantially decreased in the zona reticularis between the ages of 5-10 years. In contrast, Cyt-b₅ expression remained relatively low in the zona fasciculata and glomerulosa but substantially increased in the zona reticularis from the age of five. Therefore, 3 β HSD expression is low in the zona reticularis, which specifically expresses Cyt-b₅, whilst conversely, Cyt-b₅ expression is low in the zona fasciculata and zona glomerulosa which specifically expresses 3 β HSD.

Nevertheless, it is believed that the adrenal cortex contributes significantly to the level of circulating A4, which requires the coexpression of 3 β HSD, CYP17 and Cyt-b₅. Yet these three proteins are not coexpressed in significant levels in either of the zones of the human adrenal cortex. Nakamura et al. (2011) recently identified a layer of adrenocortical cells located between the zona fasciculata and zona reticularis that coexpress 3 β HSD, CYP17 and Cyt-b₅ and postulated this layer of cells to be the

primary site of A4 production in the adrenal. While Cyt-b₅ is vital in the stimulation of the 17,20 lyase reaction of CYP17, the stimulation of 3 β HSD by Cyt-b₅ may be equally relevant in the production of A4 by these cells, particularly as 17-OHPROG is a poor substrate for the 17,20 lyase reaction in humans and ruminants. This seems plausible as the largest increase in activity for ovine 3 β HSD in the presence of Cyt-b₅ was observed towards DHEA with a comparatively small increase in activity towards 17-OHPREG. Thus, the selective stimulation of CYP17 lyase activity and 3 β HSD activity towards 17-OHPREG and DHEA respectively would facilitate efficient synthesis of A4 in these cells.

Still, as the stimulation of 3 β HSD activity by Cyt-b₅ is only between 15 and 25%, a relatively small increase in comparison to the \approx 10-fold augmentation of CYP17 lyase activity by Cyt-b₅, the physiological relevance thereof in the production of A4 may be questionable. A recently published mathematical model simulating the network of reactions catalyzed by 3 β HSD and CYP17, however, revealed that small increases in 3 β HSD activity result in a substantial increase in A4 production, with optimal A4 production being reached within a comparatively small activity window. Furthermore, the model revealed that increasing the activity of 3 β HSD beyond a certain point paradoxically reduces A4 production as significantly more PREG and 17OHPREG are converted to PROG and 17OHPROG, neither of which are precursors for A4 (Nguyen et al., 2012). These data therefore demonstrated that a small increase in 3 β HSD activity has the potential to increase A4 production substantially. Taken together, these data suggests that during adrenal steroidogenesis the augmentation of 3 β HSD activity by Cyt-b₅ may facilitate the production of A4, a vital precursor of active sex steroids, providing physiological relevance to this novel discovery.

In addition to the adrenal cells described above, 3 β HSD and Cyt-b₅ are also coexpressed in other prominent steroidogenic organs such as the kidneys, testis and ovaries, suggesting a possible role for the augmentation of 3 β HSD activity by Cyt-b₅ in these tissues (Rheaume et al., 1991; Quinkler et al., 2003; Dharia et al., 2004; Simard et al., 2005). Precisely how augmentation of 3 β HSD activity by Cyt-b₅ would influence steroidogenesis in these tissues is, however, uncertain due to the unique qualities of the 3 β HSD family of enzymes. These enzymes are usually present in multiple isoforms within the same species, with each isoform being the product of a distinct gene. These isoforms are expressed in a tissue specific manner and vary in catalytic activity as well as substrate and co-factor specificity. For example, in humans two 3 β HSD isoforms have been characterized, namely 3 β HSD type I and II. The type I enzyme is predominantly expressed in the placenta, skin and breast tissue whilst the type II enzyme is almost exclusively expressed in the adrenals, ovaries and testis. These isoforms exhibit significant differences in catalytic activity as well as substrate and co-factor specificity (Labrie et al., 1992; Payne and Hales, 2004; Simard et al., 2005). As shown in chapter 4, Cyt-b₅ augmented 3 β HSD

activity in a species and substrate specific manner. It is plausible that Cyt-b₅ could differentially augment the activity of different 3 β HSD isoforms, thereby uniquely influencing the steroidogenic outcome in the tissues that express these isoforms. The influence of 3 β HSD augmentation by Cyt-b₅ may be tissue specific, depending on the 3 β HSD isoform present and its specific catalytic characteristics in relation to those of other steroidogenic enzymes which may compete for steroid substrate in these tissues.

Furthermore, the majority of steroidogenic enzymes involved in steroidogenesis are localized to the smooth ER membrane. Yet 3 β HSDs are unique in that they show dual sub-cellular localization between the ER membrane and mitochondrial membrane, with the degree of distribution between these varying between different tissues and species (Payne and Hales, 2004; Simard et al., 2005; Miller and Auchus, 2011). As the only form of Cyt-b₅ known to influence steroidogenesis is localized to the ER membrane (Porter, 2002; Schenkman and Jansson, 2003; Pandey and Miller, 2005) and the augmentation of 3 β HSD activity by Cyt-b₅ occurs via an allosteric mechanism, variations in the extent to which 3 β HSD is distributed to the ER membrane would significantly influence the degree to which 3 β HSD activity is augmented by Cyt-b₅. Consequently, the influence of 3 β HSD augmentation by Cyt-b₅ on steroidogenesis may also vary substantially between different tissues due to variations in the compartmentalization of these proteins within different steroidogenic cells. Indeed, Conley et al (2011) recently highlighted the importance of considering the compartmental expression of steroidogenic enzymes when comparing the production of androgens and estrogens in different tissues. The authors showed that intra-cellular compartmentalization can substantially alter the steroidogenic outcome in steroidogenic cells without requiring any alteration in enzyme expression or activity. Clearly more research is needed to determine the physiological relevance of 3 β HSD augmentation by Cyt-b₅ in the various tissue types in which these proteins are expressed.

In conclusion, the data obtained in this study demonstrated the key role of 3 β HSD during adrenal steroidogenesis and how variations in 3 β HSD activity may ultimately impact the production of active steroid hormones. Using the South African Angora goat as model we showed that differences in 3 β HSD activity significantly impact the competition between 3 β HSD and CYP17 for PREG and 17-OHPREG. This in turn alters the flux of steroid intermediates through the steroidogenic pathways leading to a significant reduction in the synthesis of the cortisol precursor 17-OHPROG. Consequently, these finding implicated 3 β HSD as an additional factor in causing hypocortisolism in the South African Angora goat. In addition, we also identified a novel interaction between 3 β HSD and Cyt-b₅ that augments 3 β HSD activity in a substrate and species specific manner. This interaction was shown to be allosteric in nature causing an increase in the affinity of 3 β HSD for NAD⁺, resulting in an increase in

the overall rate of catalysis. This represents a significant discovery in the field of steroid biochemistry as it identified a previously unknown mechanism through which steroidogenesis can be altered or regulated in different steroidogenic tissues. This study has therefore created a strong foundation on which future studies can build in order to determine how alteration of 3 β HSD activity by Cyt-b₅ influences steroidogenesis in different steroidogenic tissues.

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